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(57) Abstract

The present invention is directed to certain block copolymers of alkylethers linked to biologically active agents. More specifically, the invention relates to a conjugate between a biologically active agent and a block copolymer comprising: a biologically active agent covalently linked to a polymer comprising an A-type linear polymeric segment of relatively hydrophilic character, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500 and a B-type linear polymeric segment of relatively hydrophobic character, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80 % of the linkages joining the repeating units for each said polymeric segment comprise an ether linkage. These block copolymers, when covalently linked with biologically active agents, can stabilize such agents, facilitate their entry into cells, enhance transport across histohematic barriers and, where the target cell has developed mechanisms to reduce the cell's sensitivity to the agent, substantially overcome such resistance. Despite the covalent attachment of a large substituent, such linked agents retain substantial activity.

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POLYMER LINKED BIOLOGICAL AGENTS

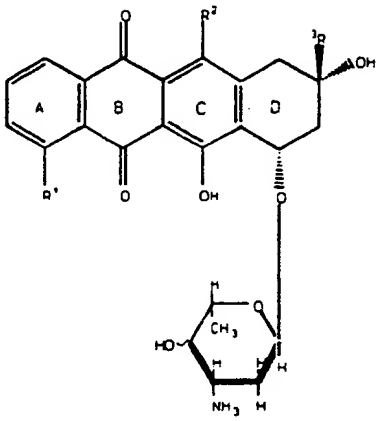
This application is a continuation-in-part of U.S. Application No. 08/342,079, filed November 18, 1994, titled "Polymer Linked Biological Agents", and is incorporated herein by reference.

The present invention is directed to certain block copolymers of alkylethers linked to biologically active agents. More specifically, the present invention relates to the discovery that certain block copolymers when covalently linked with biologically active agents can stabilize such agents, facilitate their entry into cells, enhance transport across histohematic barriers, enhance the potency of agents that interact with cell surface receptors and, where the target cell has developed mechanisms to reduce the cell's sensitivity to the agent, substantially overcome such resistance. Despite the covalent attachment of a large substituent, such linked agents can retain substantial activity.

Many drugs or other biological agents are difficult to deliver to the target tissue or organ because of undesirable biodistribution of these agents, their lability, poor membrane transport properties, and/or low efficacy of transport into target tissue or organ (which low net transport may be due to efficient, energy dependent export of the agent). Specifically, biological agents bind with serum proteins, and/or are imported by liver, other organs or white blood cells, leading to a decrease in effective concentration. Furthermore, metabolism of these agents can result in rapid clearance and in the formation of toxic metabolites that can cause side effects. In some cases the biological agents are not transported efficiently into the target tissues (e.g., cancer tumors), or cannot penetrate across histohematic barriers, such as the blood brain barrier, that isolate the target tissues or organs. In many cases the target cells develop mechanisms to reduce the cell sensitivity to the biological agents (e.g. multiple drug resistance), significantly decreasing the therapeutic effect of these agents.

Agents exhibiting such delivery problems are exemplified by many of the anti-neoplastic agents, including vinca alkaloids such as vincristine and vinblastine, mitomycin-type antibiotics such as mitomycin C and N-methyl mitomycin C, bleomycin-type antibiotics such as bleomycin A_2, antifolates such as methotrexate, aminopterin, and dideaza-tetrahydrofolic acid, colchicine,

paclitaxel, anthracycline antibiotics and others. The anthracycline antibiotics exemplify drugs having delivery problems due to low stability, the development of drug resistance in the target tissue, or rapid metabolism. These antibiotics typically include a fused tetracycline aglycone ring system joined at the 7-position to daunosamine. They include, for instance, the compounds represented by the formula:



wherein R^1 is hydroxy or methoxy; R^2 is hydrogen or hydroxy; and R^3 is ethyl, acetyl, hydroxyacetyl, or an ester of hydroxyacetyl. These tetracycline antibiotics, like many anti-neoplastic agents, are believed to act by intercalating between the planar aromatic ring structures of DNA, thereby interfering with DNA replication. See, Neidle and Waring, *Molecular Aspects of Anti-Cancer Drug Action*, Pitman Press, 1983. Neoplastic cells are generally particularly susceptible, since they are actively replicating and thus synthesizing replica copies of their DNA.

A number of efforts to stabilize such drugs have been undertaken. For instance, Page and Alakhov, Proc. Ann. Meet. Am. Assoc. Cancer Res. (1992) 33:A3302, solubilize daunorubicin in a micellar solution of poly(oxyethylene)-poly(oxypropylene) to achieve a substantial increase in cytotoxicity against drug resistant transformed cells. Yokoyama et al. synthesized a conjugate between poly(ethyleneglycol)-poly(aspartic acid) block copolymer and multiple adriamycin molecules. *Cancer Res.* 51:3229-3296 (1991). In this work, doxorubicin molecules were attached by amide bonds to a number of the carboxylic acid groups of the copolymers. The conjugate was significantly less active on a mole doxorubicin basis versus the unconjugated drug. However, Yokoyama et al. concluded that the conjugate could be safely administered at doses where the

free drug would be too toxic, and, at such elevated doses, was more effective in prolonging the survival of mice injected with tumors.

Other drug conjugates have been prepared in an effort to stabilize a drug. For instance, Pratesi et al. conjugated doxorubicin with poly-L-aspartic acid, each polymer of the conjugate attached to multiple doxorubicin molecules by ester bonds. *Br. J. Cancer*, 52:841-848, 1985. Hoes et al. coupled doxorubicin with poly- (α-L-glutamic acid) via various peptide linker/spacer groups. *J. Controlled Release*, 2:205-213, 1985. These authors reported a complex relationship between effectiveness and the type of linker used. Hoes et al. also emphasized the probable importance of carrier degradation *in vitro* to the appearance of doxorubicin activity. Duncan et al. reported linking doxorubicin to N-(2-hydroxypropyl)methyacrylamide copolymers via degradable and non-degradable linkers. *J. Controlled Release*, 10:51-63, 1989. The drug linked via degradable linker was effective, while that linked via non-degradable linker was devoid of activity.

SUMMARY OF THE INVENTION

The present invention relates to a class of covalent delivery vehicles that are structurally different from those discussed above. Conjugates with biologically active agents according to the invention have been found to retain effectiveness, to be stable in solution, to have effectiveness against cells that have been selected for drug-resistance that is essentially equal to their effectiveness against non-selected cells, and to remain in circulation for substantially longer periods than do the unmodified agents.

In one aspect, the invention relates to a drug or other biological agent delivery system. However, the invention can also be used to deliver a biological agent into a cell in vitro for diagnostic purposes. This purpose could include, for example, delivering an enzyme substrate capable of generating a fluorescent product into the cytoplasm of a cell. The enzyme activity in a cell can then be determined spectrophotometrically, and even used as a cell sorting criterion. The invention can also be used in cell culture to deliver an effector molecule such as a hormone that is necessary or facilitative for maintaining a given cell line in culture or for inhibiting the growth of a competing cell line.

The invention is described below with reference to the fragmental constants developed by Hansch and Leo. See Hansch and Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley*, New York, 1979; James, *Solubility and Related Properties*, Marcel Dekker, New York, 1986, pp. 320-325. These constants were developed for use in estimating the contribution of a portion of a molecule to the tendency of the molecule to partition between the phases formed by octanol-water mixtures. These constants are generally referred to as Hansch-Leo fragmental partition constants (hereinafter "Hansch-Leo fragmental constants").

The invention relates to a conjugate between a biologically active agent and a block copolymer comprising:

a biologically active agent covalently linked to a block copolymer comprising an A-type linear polymeric segment of relatively hydrophilic character, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500 and a B-type linear polymeric segment of relatively hydrophobic character, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units for each said polymeric segment comprise an ether linkage. In a preferred embodiment, the block copolymer comprises a polymer of formulas

A-B-A′, A-B, B-A-B′, or L(R¹)(R²)(R³)(R⁴)

(I) (II) (III) (IV)

wherein A and A′ are A-type linear polymeric segments, wherein B and B′ are B- type linear polymeric segments, and wherein R^1, R^2, R^3 and R^4 are either block copolymers of formulas (I), (II) or (III) or hydrogen and L is a linking group, with the proviso that no more than two of R^1, R^2, R^3 or R^4 are hydrogen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A - 1D show the biodistribution over time of various tritium-labeled block copolymers in blood, lever and spleen.

Figure 1E shows the pharmacokinetics of tritium-labeled polyethylene glycol.

Figure 2 shows the partitioning of various tritium-labeled copolymers between erythrocytes, lymphocytes and plasma.

Figures 3A - 3E show a comparison of the concentration of [^{3}H]-Pluronic P85 in various tissues or in blood when administered *i.v.* or orally.

DETAILED DESCRIPTION

An application titled "POLYNUCLEOTIDE COMPOSITIONS", Docket No. 313257-102, with Alexander Victorovich Kabanov, Valery Yulievich Alakhov and Sergey V. Vinogradov as named inventors, Patent Application Serial No. 08/342,209 was filed November 18, 1994. A continuation-in-part application, Docket No. 313257-102A is being filed concurrently with this application. The entire disclosure of these applications are incorporated herein by reference.

The degree of polymerization of the blocks of formulas (I), (II) or (III) can be between about 5 and about 400. Preferably, the degree of polymerization is between about 5 and about 200, more preferably, between about 5 and about 80. Preferably, the biologically active group is linked to at least one end group of the copolymers.

Biological agents are linked to the copolymers at one or more of the terminal ends. The repeating units that comprise the blocks will generally have molecular weight between about 30 and about 500, preferably between about 30 and about 100, more preferably between about 30 and about 60. Generally, in each of the blocks, at least about 80% of the linkages between repeating units will be ether linkages, preferably, at least about 90% will be ether linkages, more preferably, at least about 95% will be ether linkages. Ether linkages, for the purposes of this application, encompass glycosidic linkages when A-type or B-type blocks include carbohydrate. However, in one aspect, simple ether linkages are preferred.

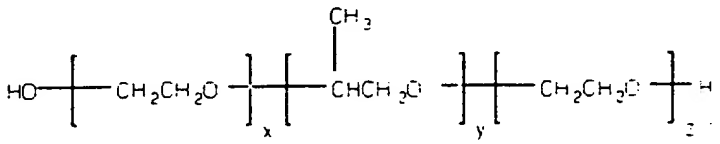
Preferably, all of the repeating units that comprise A-type blocks shall have a Hansch-Leo fragmental constant of less than about -0.4, more preferably, less than about -0.5, still more preferably, less than about -0.7. Preferably, all of the

repeating units that comprise B-type blocks shall have a Hansch-Leo fragmental constant of about -0.30 or more, more preferably about -0.20 or more.

The linkage between the biological agent and the block copolymers can be a direct linkage between a functional group at a terminal of the polymer and functional groups on the agent. Alternately, the linkage can be mediated by a bifunctional linking group X suitable for forming covalent attachments to a terminal groups of the polymer and a group on the agent. Since the linkages between repeating units of the polymer are predominately ether linkages, it will typically be the case that the terminal functionality of the polymer will be a hydroxide. Such hydroxides can be converted to other functional groups such as, for example, aldehydes, ketones, amino groups, or carboxylic acids. When the biological agent is an anthracyline, the functional group that will be linked will typically be an amino group. Generally, the linking group X will have molecular weight of no more than about 300, more typically, no more than about 150. The linking group, in some instances, is still smaller. For instance, a terminal hydroxyl on the copolymer can be modified to create an oxycarbonyl ester. This terminal ester can then be reacted with an amino group from a biologically active group to form a -O-C(O)-NH- linkage, with the -C(O)- radical serving as the linking group.

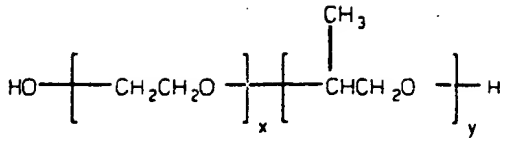
Linker L will most typically be a α,ω-di-aminoalkyl group. However, any number of multifunctional linking groups may be utilized. Generally, linking group L will have molecular weight of no more than about 600, more typically, no more than about 300.

The copolymers of the present invention are exemplified by the block copolymers having the formulas:



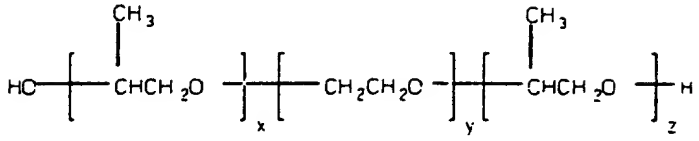
(V)

or,



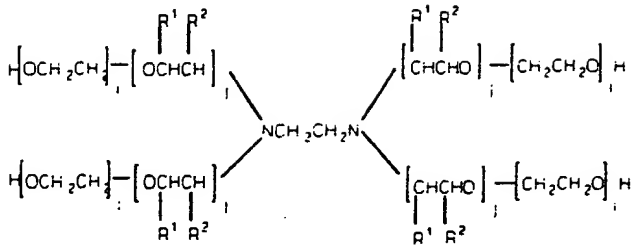
(VI)

or,



(VII)

or,

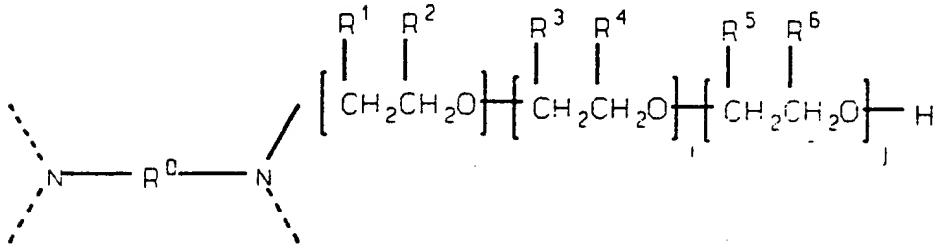


(VIII)

in which x, y, z, i and j have values from about 5 to about 400, preferably from about 5 to about 200, more preferably from about 5 to about 80, and wherein for each R^1, R^2 pair, one shall be hydrogen and the other shall be a methyl group. Formulas (V) through (VII) are oversimplified in that, in practice, the orientation of the isopropylene radicals within the B block will be mixed. This mixed orientation is indicated in formula (VIII). Such poly(oxyethylene)-poly(oxypropylene) compounds have been described by Santon, *Am. Perfumer Cosmet.*, 72(4):54-58 (1958); Schmolka, *Loc. cit.* 82(7):25-30 (1967); *Non-ionic Surfactants*, Schick, ed. (Dekker, NY, 1967), pp. 300-371. A number of such compounds are commercially available under such generic trade names as "poloxamers," "pluronics" and "synperonics." Pluronic polymers within the B-A-B formula are often referred to as "reversed" pluronics, "pluronic R" or "meroxapol." The "polyoxamine" polymer of formula (VIII) is available from BASF (Wyandotte, MI)

under the tradename Tetronic™. The order of the polyoxyethylene and polyoxypropylene blocks represented in formula (VIII) can be reversed, creating Tetronic R™, also available from BASF. See, Schmolka, *J. Am. Oil Soc.*, 59:110 (1979). Polyoxypropylene-polyoxyethylene block copolymers can also be designed with hydrophilic blocks comprising a random mix of ethylene oxide and propylene oxide repeating units. To maintain the hydrophilic character of the block, ethylene oxide will predominate. Similarly, the hydrophobic block can be a mixture of ethylene oxide and propylene oxide repeating units. Such block copolymers are available from BASF under the tradename Pluradot™. Polymers of the "Pluronic" type are known to be non-immunogenic and non-toxic.

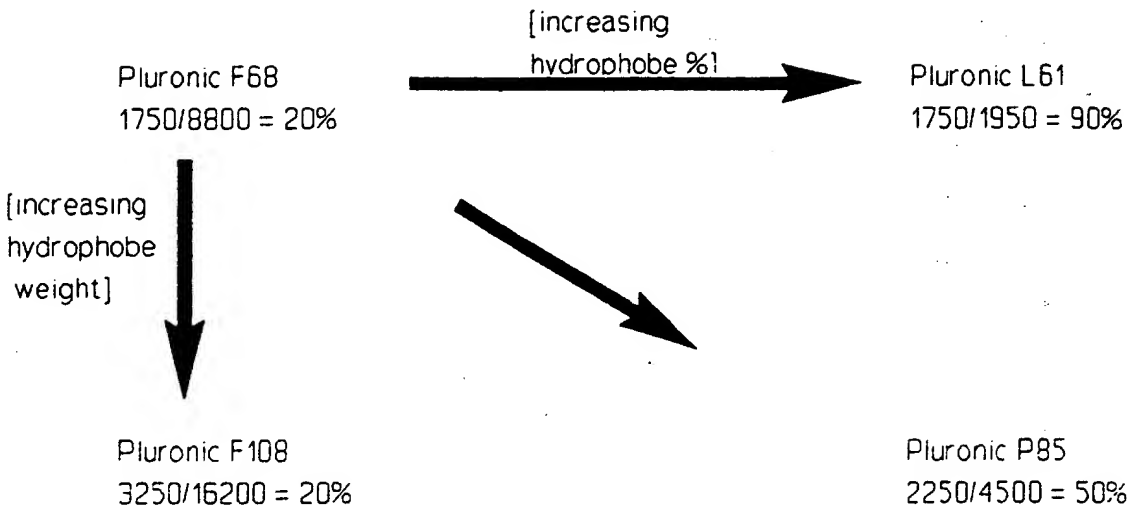
The diamine-linked pluronic of formula (VIII) can also be a member of the family of diamine-linked polyoxyethylene-polyoxypropylene polymers of formula:



(IX)

wherein the dashed lines represent symmetrical copies of the polyether extending off the second nitrogen, R^0 is an alkylene of 2 to 6 carbons, a cycloalkylene of 5 to 8 carbons or phenylene, for R^1 and R^2, either (a) both are hydrogen or (b) one is hydrogen and the other is methyl, for R^3 and R^4 either (a) both are hydrogen or (b) one is hydrogen and the other is methyl, if both of R^3 and R^4 are hydrogen, then one R^5 and R^6 is hydrogen and the other is methyl, and if one of R^3 and R^4 is methyl, then both of R^5 and R^6 are hydrogen.

The schematic below is helpful in understanding the relationship between the hydrophobe percentage and the hydrophobe weight of a copolymer and various aspects of the present invention that are discussed further below. In the schematic, the weight of the hydrophobe (poly(oxypropylene)) and of the copolymer are shown directly under each identified copolymer. Adjacent to these values are the hydrophobe percentage values for each copolymer.



These hydrophobe weights and hydrophobe percentages determine the hydrophobic-lipophilic balance of the copolymers, the *cmc* and radius of block copolymeric micelle in aqueous solution. Mortensen, K. & Pedersen, S. *Macromolecules,* 26, 805. 1993; Alexandris, et al. *Macromolecules,* 27, 2414, 1994; Kabanov, et al. *Macromolecules.,* 28, 2303, 1995. These parameters affect cell transport properties of conjugated drugs, their pharmacokinetics and biodistribution, *in vivo* and *in vitro* activity, and toxicity. In each specific case the choice of preferable copolymers is also governed by the choice of the target cell and tissue where the biological agent has to be delivered as well as by the specific mechanisms of its intracellular traffic, and the molecular target of the conjugated drug within a cell.

In certain embodiments, for instance where it is desirable to maintain a longer effective concentration of conjugate or to increase the partitioning ratio between tissue and blood, preferably the hydrophobe weight is at least about

1700 and the hydrophobe percentage is at least about 20%, preferably at least about 35%, more preferably at least about 50%; or the hydrophobe weight is at least about 2300 and the hydrophobe percentage is at least about 20%, preferably at least about 35%, more preferably at least about 50%. Such polymeric components of a conjugate, wherein the hydrophobe percentage is no more than about 50% are particularly suitable for use in treating organ disorders such as hepatic and biliary disorders including viral, microbial and cancerous disorders. In other embodiments, for instance where it is desirable to increase the partitioning ratio between tissue and blood, preferably the hydrophobe percentage of the copolymer of the composition is at least about 50% more preferably, at least about 60%, yet more preferably at least about 70%. Such conjugates are also suitable for use in treating organ disorders such as hepatic and biliary disorders including viral, microbial and cancerous disorders and for use in targeting lymphocytes for therapy with the conjugate. In other embodiments, for instance where it is desirable to increase the circulating half-life in blood (as can be useful in the treatment of certain solid tumors and microbial infections), preferably the hydrophobe percentage of the copolymer of the composition is at most about 50%, preferably at most about 30%.

It has been observed that the pharmacokinetics of several of block copolymers show oscillations in blood or tissue concentrations evidencing that the polymers recycled or recirculated, adding to their pharmacological half-life. This phenomenon is important to the design of the long lasting copolymer-linked biological agents. Specifically, due to recirculation behavior, the therapeutically effective concentration of conjugated biological agents can be maintained for a longer time after a single-dose injection, which in the case of unconjugated agents is usually achieved only by using sustained release systems, or multiple dose administration. The appearance of recirculation, the amplitude, period and decay of the oscillations are dependent of the lengths of the hydrophobic/hydrophilic properties and the lengths of the blocks.

The characteristics of a number of pluronics, described with reference to formula (IX), are as follows:

Copolymer	Hydro-phobe weight	CMC (% w/v)	Hydrophobe percentage
Pluronic L61	1750	0.0003	90
Pluronic L64	1750	0.002	60
Pluronic F68	1750	4-5	20
Pluronic P85	2250	0.005 - 0.007	50
Pluronic F127	4000	0.003 - 0.005	30
Pluronic F108	3250	.003 - 0.007	20

These CMC values were determined by the surface tension method described in Kabanov et al., *Macromolecules* 28: 2303-2314, 1995.

Additional specific poly(oxyethylene)-poly(oxypropylene) block copolymers relevant to the invention include:

Pluronic	Hydrophobe Weight	Hydrophobe Percentage
L31	950	90%
F35	950	50%
L42	1200	80%
L43	1200	70%
L44	1200	60%
L62	1750	80%
L63	1750	70%
L64	1750	60%
P65	1750	50%
L72	2050	80%
P75	2050	50%
L81	2250	90%
P84	2250	60%
F87	2250	30%
F88	2250	20%
L92	2750	80%
F98	2750	20%
L101	3250	90%
P103	3250	70%
P104	3250	60%
P105	3250	50%
F108	3250	20%
L121	4000	90%
L122	4000	80%

L123	4000	70%
F127	4000	30%
10R5*	1000	50%
10R8	1000	20%
12R3	1200	70%
17R2	1700	80%
17R1	1700	90%
17R2	1700	80%
17R4	1700	60%
17R8	1700	20%
22R4	2200	60%
25R1	2500	90%
25R2	2500	80%
25R4	2500	60%
25R5	2500	50%
25R8	2500	50%
31R1	3100	90%
31R2	3100	80%
31R4	3100	60%

*All copolymers above this conform to formula (V), this copolymer and those below conform to formula (VII).

At high concentrations, block copolymers can be toxic to the liver, kidney or other cells of a subject. *See, BASF Corp., Pluronic Material Safety Data Sheet and Drug Master Files*. The toxicity of block copolymers typically increases with the hydrophobicity parameters of block copolymers. Accordingly, toxicity is a factor in the selection of a block copolymer for use in a conjugate that will be delivered at high concentration.

Those of ordinary skill in the art will recognize, in light of the discussion herein, that even when the practice of the invention is confined to poly(oxyethylene)-poly(oxypropylene) block copolymers, the above exemplary formulas are too confining. An important feature of an A-type block is that the average Hansch-Leo fragmental constant of the repeating units in the block be about -0.4 or less. Thus, the units making up the block need not consist solely of ethylene oxide. Similarly, not all of the B-type block need be comprised solely of propylene oxide units. Instead, the blocks may incorporate repeating units other than those defined in formulas (V) - (VIII), so long as the general parameters of the A-type and B-type segments are maintained. Thus, in the

simplest of xamples, at least one of the repeating units in block A might be substituted with a side chain group.

In another aspect, the invention relates to a conjugate between a biologically active agent and a block copolymer of formula (I), (II), (III) or (IV), wherein the A and B-type blocks are substantially made up of repeating units of formula -O-R-^{5}, where R^5 is

(1) $-(CH_2)_n-CH(R^6)-$, wherein n is an integer from 0 to about 5 and R^6 is hydrogen, cycloalkyl having 3-8 carbon atoms, alkyl having 1-6 carbon atoms, phenyl, alkylphenyl wherein the alkyl has 1-6 carbon atoms, hydroxy, hydroxyalkyl wherein the alkyl has 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylcarbonyl group having 2-7 carbon atoms, alkoxycarbonyl wherein the alkoxy has 1-6 carbon atoms, alkoxycarbonylalkyl wherein the alkoxy and alkyl each independently has 1-6 carbon atoms, alkylcarboxyalkyl wherein each alkyl group has 1-6 carbon atoms, aminoalkyl wherein the alkyl group has 1-6 carbon atoms, alkylamine or dialkylamino wherein each alkyl independently has 1-6 carbon atoms, mono- or di-alkylaminoalkyl wherein each alkyl independently has 1-6 carbon atoms, chloro, chloroalkyl wherein the alkyl has from 1-6 carbon atoms, fluoro, fluoroalkyl wherein the alkyl has from 1-6 carbon atoms, cyano, cyano alkyl wherein the alkyl has from 1-6 carbon atoms or carboxyl group,

(2) a carbocyclic group having 3-8 ring carbon atoms, wherein the group can be for example, cycloalkyl or aromatic groups, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents, or

(3) a heterocyclic group, having 3-8 ring atoms, which can include heterocycloalkyl or heteroaromatic groups, which can include from 1 to 4 heteroatoms selected from the group consisting of oxygen, nitrogen, sulfur and mixtures thereto, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino

having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents.

Preferably, n is an integer from 1 to 3. The carbo cyclic or heterocyclic groups comprising R^5 preferably have 4-7 ring atoms, more preferably 5-6. Heterocycles preferably include 1-2 heteroatoms, more preferably, the heterocycles have one heteroatom. Those of ordinary skill will recognize that the monomers required to make these polymers are synthetically available. In some cases, polymerization of the monomers will require the use of suitable protective groups, as will be recognized by those of oridinary skill in the art. Preferably, the heterocycle is a carbohydrate or carbohydrate analog. Generally, the A and B-type blocks are at least about 80% by weight comprised of $-OR^5-$ repeating units, more preferably at least about 90%, yet more preferably at least about 95%.

In another aspect, the invention relates to a conjugate between a biologically active agent and a block copolymer of formulas (I), (II), (III) or (IV), wherein the A and B blocks consist essentially of repeating units of formula $-O-R^7-$, wherein R^7 is a C_1 to C_6 alkyl group. Generally, the A and B-type blocks are at least about 80% by weight comprised of $-OR^5-$ repeating units, more preferably at least about 90%, yet more preferably 95%. Preferably, the A-type blocks are substantially comprised of ethylene oxide repeating units, meaning that they are at least about 80% by weight composed of ethylene oxide repeating units. More preferably the A-type blocks are at least about 90%, yet more preferably 95%, comprised of ethylene oxide repeating units. Preferably, the B-type blocks are substantially comprised of propylene oxide repeating units, meaning that they are at least about 80% by weight composed of propylene oxide repeating units. More preferably the B-type blocks are at least about 90%, yet more preferably 95%, comprised of propylene oxide repeating units.

The Hansch-Leo estimate of the octanol-water partitioning coefficient (P) for an organic molecule is calculated by the following formula:

$$L\ g\ P = \Sigma\ a_n f_n + \Sigma\ b_m F_m$$

where the f_n values are the fragmental constants for the different groups in the molecule, the a_n values are the number of any type of group in the molecule, the

F_m values are factors for certain molecular features such as single bonds or double bonds between groups for which fragmental constants have been assigned, and the b_m values are the number of any such molecular feature. For instance, the Hansch-Leo fragmental constant for an ethylene oxide repeating unit ($-CH_2CH_2O-$) would be:

$$2f_C + 4f_H + f_O + (4-1)F_b = 2(0.20) + 4(0.23) + (-1.82) + 3(-0.12) = -0.86$$

The Hansch-Leo fragmental constant for a propylene oxide ($-CH_2CH_2(CH_3)O-$) repeating unit would be:

$$2f_C + f_{CH_3} + 3f_H + f_O + (4-1)F_b = 2(0.2) + 0.89 + 3(0.23) + (-1.82) + 3(-0.12) = -0.2$$

Those of ordinary skill in the art will recognize that the Hansch-Leo approach to estimating partition constants, in which approach the Hansch-Leo fragmental constants are applied, does not yield precisely the empirical partition constant. See Hansch and Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley*, New York, 1979; James, *Solubility and Related Properties*, Marcel Dekker, New York, 1986, pp. 320-325. However, the approach is precise enough to define the hydrophobicity features of the polymeric delivery vehicle.

While not wishing to be confined to theory, it is believed that one reason the conjugate of the present invention retains substantial biological activity is the flexibility of the ether linkages that are the predominate linker between the repeating units of the block copolymer of the invention. Because of the flexibility of the conjugate to the agent, it will have less tendency to block access of the agent to its intracellular site of action than would less flexible polymeric adducts, such as those reported by Hoes et al., *J. Controlled Release*, 2:205-213, 1985.

It is not critical to the present invention that the conjugate of the present invention be present in a micelle. Micelles are supramolecular complexes that form in aqueous solutions of certain amphipathic molecules due to the microphase separation of the nonpolar segments of the amphipathic molecules. Micelles form when the concentration of the amphipathic molecule reaches, for a given temperature, a critical micellar concentration ("CMC") that is characteristic of the amphipathic molecule.

The block copolymers utilized in the invention, when at a concentration above the CMC, will typically form micelles of from about 10 nm to about 100 nm in diameter. Such micelles will generally include from about 10 to about 300 block copolymers. By varying the sizes of the hydrophilic and hydrophobic portions of the block copolymers, the tendency of the copolymers to form micelles at physiological conditions can be varied. The micelles have a dense core formed by the water insoluble repeating units of the B blocks, and a hydrophilic shell formed by the A blocks. The micelles have translational and rotational freedom in solution, and solutions containing the micelles generally have low viscosity similar to that of water. Micelle formation typically occurs at concentrations from about 0.001 to 5% (w/v).

At high concentrations, some of the block copolymers utilized in the invention will form gels. These gels are viscous systems in which the translational and rotational freedom of the copolymer molecules is significantly constrained by a continuous network of interactions among copolymer units. In gels, microsegregation of the B block repeating units may or may not occur. To avoid the formation of gels, polymer concentrations will preferably be below about 15% (w/v), more preferably below about 10%, still more preferably below about 5%.

Due to the amphipathic character of the block copolymers of the conjugate, when the concentration of conjugate is sufficient, micelles will form. Under some circumstances, it is anticipated that the presence of micelles will be desirable. For instance, it may be desirable to incorporate an unconjugated agent, which agent can be the same or different from that conjugated to copolymer, in a micelles for coordinated administration with the conjugate. This approach would allow fine tuning of the pharmacokinetics and biodistribution of the agent. Drug dissolved in pluronic micelles would be expected to be more quickly available at the site of action. However, it would not be expected to remain available over the substantially longer time periods over which the conjugate would remain available. Thus, in some instances, it can be desirable to have coadminister conjugate and unmodified bioactive agent, which may be

dissolved in either conjugate or a mixture of conjugate and one or more block copolymer according to one of formulas (I), (II), (III) or (IV).

Biological agents in the conjugate or dissolved in block co-polymer micelles have greater stability, resistance to degradative processes, resistance to nonspecific absorption and specific absorption onto carrier molecules such as bovine serum albumin, and resistance to uptake by liver and white blood cells. However, the micelle carriers generally will release over time the bioactive free form of the bioactive agent and to disaggregate from the micellar form when their concentration becomes low enough. The conjugate of the present invention provides a longer-term protective carrier.

While not wishing to be limited by theory, it is believed that when free or conjugated biological agent is in a micelle, a significant route of entry into cells will be through endocytosis. When not in a micelle, it is believed that entry is more direct. In either case, the hydrophobic portion of the conjugate is believed to facilitate cross-membrane transit of the conjugate, allowing the conjugate to encounter the cytoplasm of a target cell. In the case of an endocytosed micelle, while the micelle is "inside" the cell, it is still topologically isolated from the cytoplasm. However, it is believed that, in the harsh environment of the lysosomes into which endocytosed material will be transported, a portion of the copolymers conjugated to the bioactive agent will dissociate from the micelles, at which point the flexible hydrophobic moiety of the conjugate can facilitate transmembrane transport.

It will in some circumstances be desirable to incorporate the conjugate into micelles to allow for the noncovalent association of targeting molecules. See, for example, Kabanov et al., *J. Controlled Release*, 22:141 (1992). The targeting molecules that can be associated with the conjugate typically have a targeting group having affinity for a cellular site and a hydrophobic group. If the conjugate is formulated so that it will form micelles, by itself or in conjunction with other amphipathic molecules, the targeting molecule will spontaneously associate with the micelles and be "anchored" thereto through the hydrophobic group. These targeting molecules will typically comprise about 10% by weight or less of the conjugate or block copolymer in a composition.

In the targeting molecule, the hydrophobic group can be among other things, a lipid group such as a fatty acid group. Alternately, it can be a block copolymer or other synthetic natural occurring polymer. The targeting group of the targeting molecule will frequently comprise an antibody, typically with specificity for a certain cell surface antigen. It can also be, for instance, a hormone having a specific interaction with a cell surface receptor, or a drug having a cell surface receptor. For example, glycolipids can serve as the targeting group to target a polysaccharide receptor. Note that targeting molecules can be used to facilitate intracellular transport of the polynucleotide composition, for instance transport to the nucleus, for instance by using, as targeting molecules the fusogenic peptides described be Soukchareun et al., *Bioconjugate Chem.* 6, 43, 1995 or Arar et al., *Bioconjugate Chem.* 6, 43, 1995, caryotypic peptides, or other biospecific groups providing site-directed transport into a cell (in particular, exit from endosomic compartments into cytoplasm, or delivery to nucleus).

In another embodiment of the invention, the targeting group can be attached to one end group of the copolymer, while the biological agent can be attached to one or more other end groups.

For polyethylene oxide-polypropylene oxide copolymer, the hydrophilic/hydrophobic properties, and micelle forming properties of a block copolymer are, to a certain degree, related to the value of a constant,n. The constant, n, is defined as:

$$n = (|B|/|A|) \times (b/a) = (|B|/|A|) \times 1.32$$

where |B| and |A| are the totals of the number of repeating units of polymerization of the hydrophobic and hydrophilic blocks of the copolymer, respectively, and **b** and **a** are the molecular weights for the respective repeating units. The value of n will typically be between about 0.2 and about 9.0, more preferably, between about 0.2 and about 1.5. Where mixtures of block copolymers are used, n will be the weighted average of n for each contributing copolymers, with the averaging based on the weight portions of the component copolymers. When copolymers other than polyethylene oxide-polypropylene oxide copolymers are used, similar approaches can be developed to relate the

hydrophobic/hydrophilic properties of one member of the class of polymers to the properties of another member of the class.

Biologically Active Agents

Virtually any biologically active agent (also referred to herein as biological agent or bioactive agent), i.e., an agent that can act on a cell, organ or organism, including but not limited to drugs (pharmaceuticals) to create a change in the functioning of the cell, organ or organism, including but not limited to drugs (pharmaceuticals) can be used in the conjugate of the present invention. Imaging agents, contrast agents and dyes are also biologically active agents. However, those that have low water solubility are particularly preferred. Such bioactive agents include but are not limited to anti-neoplastic agents such as paclitaxel, daunorubicin, doxorubicin, carminomycin, 4'-epiadriamycin, 4-demethoxy-daunomycin, 11-deoxydaunorubicin, 13-deoxydaunorubicin, adriamycin-14-benzoate, adriamycin-14-octanoate, adriamycin-14-naphthaleneacetate, vinblastine, vincristine, mitomycin C, N-methyl mitomycin C, bleomycin A_2, dideazatetrahydrofolic acid, aminopterin, methotrexate, cholchicine and cisplatin, antibacterial agents such as aminoglycosides including gentamicin, antiviral compounds such as rifampicin, 3'-azido-3'-deoxythymidine (AZT) and acylovir, antifungal agents such as azoles including fluconazole, polyene macrolides such as amphotericin B, and candicidin, anti-parasitic compounds such as antimonials, proteins, peptides or polypeptides such as antibodies, toxins such as diphtheria toxin, peptide hormones, such as colony stimulating factor, and tumor necrosis factors, neuropeptides, growth hormone, erythropoietin, and thyroid hormone, neurotransmitters such as acetylcholine, lipoproteins such as alpha-lipoprotein, proteoglycans such as hyaluronic acid, glycoproteins such as gonadotropin hormone, immunomodulators or cytokines such as the interferons or interleukins, hormone receptors such as the estrogen receptor, non-steroidal anti-inflammatories such as indomethacin, salicylic acid acetate, ibuprofen, sulindac, piroxicam, and naproxen, antiglaucomic agents such as timolol or pilocarpine, anesthetics such as dibucaine, nucleic acids such as thymine, polynucleotides such as DNA or RNA polymers or synthetic oligonucleotides, which may be derivatized. The conjugate of the invention is

anticipated to be particularly useful in increasing the activity of antisense DNA or RNA molecules. For the purpose of this application, enzymes are not biological agents. In one aspect of the invention, interferons will not be used as the biological agent.

Polynucleotides have been derivatized to facilitate entry into cells by covalently modifying the 5' or the 3' end of the polynucleic acid molecule with hydrophobic substituents. These modified nucleic acids generally gain access to the cells interior with greater efficiency. See, for example, Kabanov et al., *FEBS Lett.*, 259:327 (1990); Boutorin et al., *FEBS Lett.*, 23:1382-1390, 1989; Shea et al, *Nucleic Acids Res.*, 18:3777-3783, 1990. Additionally, the phosphate backbone of the polynucleotides has been modified to remove the negative charge (see, for example, Agris et al., *Biochemistry*, 25:6268 (1986); Cazenave and Helene in *Antisense Nucleic Acids and Proteins: Fundamentals and Applications*, Mol and Van der Krol, eds., p. 47 *et seq.*, Marcel Dekker, New York, 1991) or the purine or pyrimidine bases have been modified (see, for example, *Antisense Nucleic Acids and Proteins: Fundamentals and Applications*, Mol and Van der Krol, eds., p. 47 *et seq.*, Marcel Dekker, New York, 1991; Milligan et al. in *Gene Therapy For Neoplastic Diseases*, Huber and Laso, eds., p. 228 *et seq.*, New York Academy of Sciences, New York, 1994). It is believed that the these modification techniques can be used in conjunction with the present invention of conjugating the polynucleotide to the block copolymers to increase the effect of the polynucleotide on target cells. When the invention is used with polynucleotides, it will often prove useful to neutralize the phosphate backbone of the polynucleotide with a hydrophobic cation such as N-[1-(2,3-dioleyloxy)-N,N-3'-methyammoniumchloride] or with a polycation. Polycations that present cationic groups with a spacing that matches the spacing of the phosphate groups on polynucleic acid are particularly preferred. For instance, the polycations with repeating unit -NH-$CH_2CH_2CH_2$- or repeating units comprising a mixture of -NH-$CH_2CH_2CH_2$- and -NH-$CH_2CH_2CH_2CH_2$- are particularly preferred.

The conjugate of the present invention is anticipated to more readily traverse the blood-brain barrier than does the corresponding free biological agent. Accordingly, a number of neuro-acting agents are anticipated to be

useful as agents that can be conjugated according to the invention. These include, without limitation, neuroleptics such as the phenothiazines (for example compazine, thorazine, promazine, chlorpromazine, acepromazine, aminopromazine, perazine, prochlorperazine, trifluoperazine, and thioproperazine), rauwolfia alkaloids (for example, reserpine and deserpine), thioxanthenes (for example chlorprothixene and tiotixene), butyrophenones (for example haloperidol, moperone, trifluoperidol, timiperone, and droperidol), diphenylbutylpiperidines (for example pimozide), and benzamides (for example sulpiride and tiapride); tranquilizers such as glycerol derivatives (for example mephenesin and methocarbamol), propanediols (for example meprobamate), diphenylmethane derivatives (for example orphenadrine, benzotrapine, and hydroxyzine), and benzodiazepines (for example chlordiazepoxide and diazepam); hypnotics (for example zolpdem and butoctamide); beta-blockers (for example propranolol, acebutonol, metoprolol, and pindolol); antidepressants such as dibenzazepines (for example, imipramine), dibenzocycloheptenes (for example, amitriptyline), and the tetracyclics (for example, mianserine); MAO inhibitors (for example phenelzine, iproniazid, and selegeline); psychostimulants such as phenylethylamine derivatives (for example amphetamines, dexamphetamines, fenproporex, phentermine, amfeprramone, and pemoline) and dimethylaminoethanols (for example clofenciclan, cyprodenate, aminorex, and mazindol); GABA-mimetics (for example, progabide), alkaloids (for example co-dergocrine, dihydroergocristine, and vincamine); cholinergics (for example citicoline and physostigmine); vasodilators (for example pentoxifyline); and cerebro active agents (for example pyritinol and meclofenoxate).

The invention is particularly suited for stabilizing and delivering molecules that interact with a cell surface receptor. It has been observed that for such molecules the conjugate of the invention can be more effective than the unconjugated molecule, even in vitro where degradative processes are not anticipated to create substantial problems. While not wishing to be limited by theory, it is believed that this increase in effectiveness is due to the interaction of the B-type polymer block with cell membranes. Through this interaction, it is

believed that the effective affinity constant of the effector for its receptor is increased substantially.

Preferred classes of biological agents include anti-neoplastic agents, antibacterial agents, antiparasitic agents, CNS agents, immunomodulators and cytokines, toxins, neuropeptides and polynucleotides. Biological agents, such as certain drugs for which target cells tend to develop resistance mechanisms are also preferred. Particularly preferred biological agents include anthracyclines such as doxorubicin, daunorubicin, or carminomycin, vinca alkaloids, mitomycin-type antibiotics, bleomycin-type antibiotics, fluconazole, amphotericin B, paclitaxel, taxotere and related compounds and derivatives, immunomodulators and cytokines such as interleukins and TNFs, erythropoietin, and polynucleotides, especially oligonucleotides.

For non-hormone biological agents, biological agents of molecular weight less than about 50,000 are preferred. More preferred are molecular weights less than about 40,000, still more preferred are molecular weights less than about 15,000. Biological agents that act by specifically interacting with a cellular molecule are preferred.

Biological Agent - Polymer Linkages

There are a large number of methods for linking biologically active agents to a block copolymer. Examples of such methods are outlined below. The methods often recite that a first linkable group, such as an amino or a hydroxyl group, is found at the terminal of the block copolymer, while a second linkable group is associated with the biologically active agent. Those of ordinary skill will recognize that in such cases the method generally can be adapted if the first linkable group is on the agent instead of the copolymer and the second is on the copolymer. Similarly, those of ordinary skill will recognize that a number of reactions recited below as applicable to linking a certain group can also be applied to strategies for linking other groups with related reactivities.

1. Reductive Alkylation - Linking Amino and Hydroxyl

Where the block copolymer terminates in one or more hydroxyl groups, these hydroxyl groups can be utilized to link the biologically active agent to the polymer. For instance, the terminal hydroxyls can be oxidized to an

aldehydes. The aldehydes can then be reacted with amino groups that are substituents or adducts of the biologically active agent to form Schiff bases. These, in turn, can be readily reduced, preferably with sodium borohydrate to form a C-N linkage between polymer and biologically active agent. See Kabanov et al., *J. Controlled Release*, 22:141 (1992); *Meth Enzymol.*, XLVII, Hirs & Timasheff, Eds., Acad. Press, 1977.

2. Coupling Polymer and Agent Through an Acetyl Linkage

Where the block copolymer terminates in one or more hydroxyls, the hydroxyls can be reacted with bromoacetyl chlorides, forming bromoacetyl esters. The bromo groups can then be reacted with amino groups that are substituents or adducts of biologically active agents, forming -N-CH_2-C(O)O-C- linkages. *Immobilized Enzymes*, Berezin et al., eds., MGU, Moscow, 1976.

3. Imidoester Linkages

Where the block copolymer terminates in a hydroxyl, it can again be converted to a bromoacetyl ester, as above. The bromoacetyl ester can be reacted with a cyanide salt, forming a cyano intermediate. See, e.g., Sekiguchi et al., *J. Biochem.*, 85, 75 (1979; Tuengler et al., *Biochem. Biophys. Acta*, 484, 1 (1977); Browne et al, *BBRC*, 67 126 (1975); and Hunter et al., *J.A.C.S.*, 84, 3491 (1962). The cyano intermediate can then be converted to an imido ester, for instance, by treatment with a solution of methanol and HCl. The imidoester can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to create a -N-C(NH_2^+)CH_2C(O)O-C- linkage.

4. Linkage Formed Using 1,1′-carbonyl-bis-imidazole

A block copolymer terminating in at least one hydroxyl can be reacted with 1,1′-carbonyl-bis-imidazole. The intermediate so formed can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to form a -N-C(O)O-C- linkage. See Bartling et al., *Nature*, 243:342 (1973).

5. Linkage Using Cyclic Anhydrides

A block copolymer terminating in at least one hydroxyl can be reacted with e.g. succinic anhydride, to create a terminal carboxylic acid linked to the polymer via an ester bond. The acid group can be reacted with an amino group

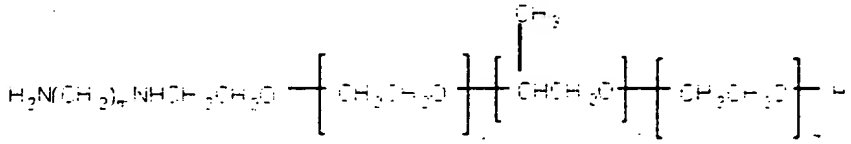
that is a substituent of or an adduct of a biologically active agent to form an amide bond. The amide formation can be directly mediated with a carbodiimide compound. Alternately, a reactive ester can be formed with the acid group and N-hydroxysuccinimide using a carbodiimide mediated reaction. The reactive ester will readily react with the amino group to form the amide. See, Means et al., *Chemical Modification of Proteins*, Holden-Day (1971).

6. Epoxide Mediated Linkages

A block copolymer terminating in at least one hydroxyl can be reacted with a bis epoxide compound such as 1,4-butanediol diglycidyl ether, to form a structure having a terminal epoxide function linked to the polymer by an ether bond. The terminal epoxide function is, in turn, reacted with an amino group that is a substituent of or an adduct of a biologically active agent to form a nitrogen-carbon linkage. Pitha et al., *Eur. J. Biochem.*, 94:11 (1979); Elling and Kula, *Biotech. Appl. Biochem.*, 13:354 (1991); Stark and Holmberg, *Biotech. Bioeng.*, 34:942 (1989).

7. Reductive Alkylation - linking amino groups.

This procedure relates to block copolymers terminating in at least one terminal amino group, such as the copolymer illustrated below:



(X)

wherein m is an integer 1 to 25, and x, y and z are as defined above. The amino group may be alkylated by incubating with glutaraldehyde and a reducing agent such as sodium cyanoborohydride. This process will generate a terminal oxygen-containing group which may be an aldehyde or, if the second aldehyde group from glutaraldehyde was reduced in the first step, a hydroxyl group. If the terminal group is a hydroxyl group, it can be oxidized to an aldehyde group with an oxidant such as sodium iodate. This aldehyde functionality can be coupled with an amine group that is a substituent of or an adduct of a biologically active

agent by reductive alkylation to form a -N-$(CH_2)_4$-N- linkage. Means and F eney, *Biochemistry*, 7:2192 (1968).

8. Isothiocyanate-Mediated Coupling

A block copolymer terminating in at least one amino group can be reacted with carbon disulfide in the presence of potassium hydroxide. Proprionyl chloride is then added to the reaction mixture. This reaction creates a terminal isothiocyanate group. The isothiocyanate group can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to create a -N-C(S)-N- linkage. See Means et al., *Chemical Modification of Proteins*, Holden-Day (1971).

9. Phosgene-Mediated Linkage

A block copolymer terminating in at least one amino group can be treated with phosgene to create reactive intermediate. The intermediate can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to yield a -N-C(O)-N- linkage. See Means et al., *Chemical Modification of Proteins*, Holden-Day (1971).

10. Dimethyl Adipimidate-Mediated Linkage

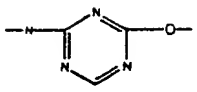
A block copolymer terminating in at least one amino group can be reacted with dimethyl adipimidate to create a reactive intermediate. The reactive intermediate can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to create a -N-C(NH_2^+)-$(CH_2)_4$-C(NH_2^+)-N- linkage. See Lowe et al., *Affinity Chromatography*, Wiley & Sons, 1974.

11. 1,6-Diisocyanohexane-Mediated Linkage

A block copolymer terminating in at least one amino group can be reacted with 1,6-diisocyanohexane to create a reactive intermediate. The reactive intermediate can then be reacted with an amino group that is a substituent of or an adduct of a biologically active agent to form a -N-C(O)NH$(CH_2)_6$NHC(O)-N- linkage. See Means et al., *Chemical Modification of Proteins*, Holden-Day (1971).

12. The Technique of Kaye et al.

The technique of Kaye et al., *Nature*, 216:514, 1967 can be used to create a



linkage between an amino group and a hydroxyl group.

The above reaction descriptions exemplify the large number of linkage strategies that can be employed to link a biologically active agent to the block copolymers of the invention. Other strategies will be apparent to those of ordinary skill in the art. Further general conjugation methods are described Means et al., *Chemical Modification of Proteins*, Holden-Day (1971); Glazer et al., *Chemical Modification of Proteins*, Elsevier, New York (1975); *Immunotechnology Catalog & Handbook*, Pierce Chemical Co.; *Polyethylene Glycol Derivatives*, Catalog, Shearwater Polymers, Inc. (1994).

The conjugate of the invention can be administered orally, topically, rectally, vaginally, by pulmonary route by use of an aerosol, or parenterally, i.e. intramuscularly, subcutaneously, intraperitoneallly or intravenously. The conjugate can be administered alone, or it can be combined with a pharmaceutically-acceptable carrier or excipient according to standard pharmaceutical practice. For the oral mode of administration, the conjugate can be used in the form of tablets, capsules, lozenges, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the conjugate can be combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added. For parenteral administration, sterile solutions of the conjugate are usually prepared, and the pH of the solutions are suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic. For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl

methylcellulose or polyvinyl alcohol, preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. For pulmonary administration, diluents and/or carriers will be selected to be appropriate to allow the formation of an aerosol.

The invention is further explained by reference to the following non-limiting examples. The examples make use of the following block copolymers of Formula II:

Block Copolymer	x	y	z
Pluronic A	25	38	25
Pluronic B	80	30	80
Pluronic C	150	56	150

Example 1 - Conjugate Synthesis

Tert-butylhypochlorite (300 μl) was added to 520 mg of Pluronic A dissolved in 15 mL of tert-butyl alcohol to initiate a reaction. The reaction was conducted at 20°C in the dark for 20 hours. The precipitated intermediate that formed during the reaction was collected by filtration and dried under vacuum. The precipitate was dissolved in 5 mL isopropanol and heated in a water bath for two hours. The intermediate recovered after evaporating the solvent contained 1.15 aldehyde groups per polymer molecule. (The aldehyde content was determined using a dinitrophenylhydrazine assay.)

33 mg of the intermediate was dissolved in 1 ml of 0.05 M borate buffer (pH 8.8 to 9.0). To this mixture was added 3.5 mg of daunorubicin and 25 mg of sodium cyanoborohydride to initiate a reductive alkylation reaction. The reaction mixture was stirred for 12 hours at 4°C. The polymeric products of the reaction were isolated by gel filtration chromatography on Sephadex LH-20, utilizing 90% aqueous isopropanol as the eluent. The isolated product contained 0.8 daunorubicin molecules per polymer molecule, as measured spectrophotometrically at 450 nm. Thin-layer chromatography confirmed the absence of any free daunorubicin in the polymeric product.

Similar conjugation efficiencies have been achieved using doxorubicin, carminomycin, 4'-epiadriamycin, 4-demethoxydaunomycin, 11-deoxydaunorubicin, 13-deoxydaunorubicin, adriamycin-14-benzoate,

adriamycin-14-octanoate, and adriamycin-14-naphthaleneacetate in place of daunorubicin.

Example 2 - Conjugate Synthesis

1 g of Pluronic B was dissolved in 5 mL of dried benzene. Molecular sieves (0.3g, 3 Å pores), were added to the solution, which was allowed to stand over night to remove residual moisture. Approximately 3 to 4 g of dried sodium carbonate and 1.5 g of freshly recrystallized 2,4,6-tricholoro-1,3,5-triazine were then added to this dried solution to initiate a chlorination reaction. This reaction mixture was stirred for 24 hours. The solution was decanted from the molecular sieves and 20 mLs of hexane were added, resulting in the precipitation of a solid reactive intermediate. The solid was collected and dissolved in 2 mL of benzene. From this benzene solution, the reaction product was again precipitated by the addition of 10 mL of petroleum ether. This precipitation procedure was repeated four more times to remove unreacted 2,4,6-trychloro-1,3,5-triazine.

1 g of this reactive intermediate was dissolved in 1 mL of benzene. A solution of 0.5 g hexamethylenediamine (1,6-hexanediamine) dissolved in 1 mL of benzene was added to the dissolved intermediate to initiate a nucleophilic substitution reaction. This reaction mixture was allowed to stand at room temperature. After 12 hours of reaction, the polymer product was isolated from the reaction mixture by gel filtration on Sephadex LH-20, using isopropanol as the eluent. The polymeric second intermediate so isolated contained 0.3 amino groups per polymer molecule, as indicated by trinitrobenzenesulfonic acid titration. The absence of hexamethylenediamine was confirmed by thin-layer chromatography. 170 mg of this second intermediate were dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.0). This solution was mixed with 160 mL of a 25% aqueous solution of glutaraldehyde to initiate the formation of Schiff base linkages between the glutaraldehyde reactant and the polymer intermediate. This reaction mixture was stirred for 12 hours at room temperature. A polymeric third intermediate was isolated from this mixture by gel filtration chromatography on Sephadex LH-20, using 90% aqueous isopropanol as the eluent.

The isolated third intermediate (30 mg) was dissolved in 1 mL of 0.05 M borate buffer (pH 8.8 to 9.0). To this solution were added 3 mg of daunorubicin and 20 mg of sodium cyanoborohydride, initiating a reductive alkylation reaction. The reaction mixture was stirred at 4°C for 12 hours. The polymeric product from this reaction was isolated by gel filtration chromatography on LH-20 resin, using a 90% aqueous isopropanol solution as the eluent. The polymeric product contained 0.3 daunorubicin molecules per polymer molecule. The absence of free daunorubicin was confirmed by thin-layer chromatography.

Example 3 - Conjugate Synthesis

The procedures of Example 2 were used except that Pluronic C was used as the starting polymer and doxorubicin was used as the biologically active agent. The product contained 0.4 doxorubicin molecules per polymer molecule.

Example 4 - Solution Stability

The conjugate between daunorubicin and Pluronic A prepared in Example 1 ("conjugate A") was dissolved in sterile phosphate-buffered saline ("PBS") (pH 7.0) at a concentration of 0.2 μM. The concentration was calculated on the basis of the daunorubicin content of the conjugate. A corresponding 0.2 μM solution of free daunorubicin was prepared as a control. Both preparations were incubated at 37°, and the dissolved concentration of daunorubicin monitored by fluorescence spectrophotometry (λ_{ex} = 485 nm). The results were as follows:

Duration of incubation (hours)	I_{585} (% of initial)	
	Conjugate A	Control
0	100	100
6	103	92
27	100	89
43	97	68
78	98	54
120	100	37

Example 5 - Cytotoxicity Towards Agent Resistant Transformed Cells

Conjugate A was dissolved in RPMI 1640 medium (ICN Biomedicals Inc., Costa Mesa, CA) to a final concentration of 1%, and then the solution was filtered through a 0.22 μm filter to remove bacterial or fungal contamination. This conjugate A solution was used to make appropriate dilutions for the cell culture experiments described below. A comparable stock solution of unconjugated daunorubicin was also made.

The cytotoxicity studies utilized the SKOV3 line of transformed cells (hereinafter "SK cells") and the SKVLB cell line derived therefrom (hereinafter "SK-resistant cells"). The SK-resistant cell line is a multi-drug resistant transformed cell line derived from the SK cell line by long term cultivation in the presence of vinblastine.

Various dilutions of conjugate A or free daunorubicin were added to cell cultures cultured in RPMI 1630 medium supplemented with 10% fetal calf serum, at 37°C, and under a 5% CO_2 atmosphere. The cells were exposed to conjugate A or free daunorubicin for one hour. After this incubation, the cells were washed three times with fresh medium. Then, the cells were cultured under fresh medium for an additional four days.

The number of viable cells for each culture was determined by standard XTT analysis, which measures the activity of mitochondrial enzymes. See, Scudievo et al., *Cancer Res.*, 48:4827 (1988). IC_{50} values (i.e., the concentration at which 50% inhibition was achieved) were determined by extrapolating from graphs plotting the number of viable cells versus the concentration of drug applied to the cells. The results were as follows:

Sample	IC_{50} (μg/mL)*	
	SK	SK-resistant
Conjugate A	2.10	2.02
Free daunorubicin	0.32	2.55

*The concentration of free daunorubicin or of daunorubicin residues incorporated into the conjugate.

Example 6 - Cytotoxicity Towards Drug Resistant Transformed Cells

The conjugate between doxorubicin and Pluronic B prepared in Example 2 ("Conjugate B") was tested for cytotoxicity by the same methods outlines above for Example 5. The results were as follows:

Sample	IC_{50} (μg/mL)	
	SK	SK-resistant
Conjugate B	37	27
Free daunorubicin	5.2	45

Example 7 - Cytotoxicity Towards Drug Resistant Transformed Cells

The cytotoxicity of Conjugate A was tested with respect to murine myeloma cell line Sp2/0 (hereinafter "Sp2") and a multi-drug resistant cell line derived therefrom by multiple passages in the presence of daunorubicin (the "Sp2-resistant cell line"). The site of toxicity was determined using the methods outlined in Example 5. The results were as follows:

Sample	IC_{50} (μg/mL)	
	Sp2	Sp2-resistant
Conjugate A	2.70	2.55
Free daunorubicin	0.22	2.60

Example 8 - Animal Toxicity

Conjugate A was injected intraperitoneally into C57B1/6 male mice at a variety of dose amounts. Ten animals were treated at each dose level. Corresponding toxicity experiments were done with unmodified daunorubicin. Animal survival was monitored daily for 14 days. From the data collected, maximum tolerated doses ("MTDs", the maximum dose that does not lead to death) and LD_{50} values were calculated. The results were as follows:

Sample	MTD (mg/kg)*	LD_{50} (mg/kg)*
Conjugate A	112	280
Control	5.0	7.0

*Concentrations measured on the basis of daunorubicin content.

Example 9A - Tissue Distribution

Conjugate A* was prepared according to the protocol of Example 1 except that a tracer amount of [^{3}H]-labelled Pluronic A was incorporated into the conjugate. Various doses of Conjugate A* dissolved in phosphate-buffered saline, ("PBS") were injected intravenously into 7 week old C57B1/6 male mice (6 animals treated for each dose level, and for each time of treatment). The animals were sacrificed either at one hour or at 19 hours after injection. Tissue samples were collected and placed in 1 mL of tissue solubilizer (available from Serva, Germany) and homogenized in the cold. The homogenates were incubated for 14 hours at room temperatures, decolorized with 50 μL of 30% hydrogen peroxide, and incubated overnight at room temperature. The radioactivity in the samples was measured by liquid scintillation counting. The results were as follows:

Organ	Drug Content (% of initial dose/organ ± SEM)	
	1 hour	19 hours
Blood	72.3 ± 2.9	11.0 ± 1.2
Liver	4.8 ± 0.3	1.28 ± 0.43
Kidney	3.2 ± 0.2	0.84 ± 0.21
Lung	0.8 ± 0.1	0.27 ± 0.06
Spleen	1.2 ± 0.3	0.17 ± 0.07
Heart	0.2 ± 0.0	0.17 ± 0.02
Brain	0.9 ± 0.2	0.14 ± 0.01

Example 9B - Polymer Pharmacokinetics and Tissue Distribution

[^{3}H]-labeled block copolymers L61, F68, P85 and F108 or [3H]-labeled poly(ethylene oxide), ("PEG"), M.W. 4000, were obtained from

Kurchatov Institute of Atomic Energy, Moscow, Russia, were administered i.v. in groups of female Balb/c mice (100 μl of 1 % solution ($2x10^7$ to 10^8 cpm) in PBS 20 g body weight). At a given time (up to 200 hours) the animals in each treatment group were sacrificed (3-4 animals per each time point). Blood and tissue samples were collected and placed in 1 ml of tissue solubilizer (available from Serva, Germany) and homogenized in the cold. The homogenates were incubated for 14 hours at room temperatures, decolorized with 50 μl of 30 % hydrogen peroxide, and incubated overnight at room temperature to allow complete dissolution of the samples. The radioactivity was measured by liquid scintillation counting. The results, for block copolymers L61, F68, P85 and F108 are presented in Figure 1A, Figure 1B, Figure 1C, and Figure 1D, respectively. The points indicated with an "x" indicate the percent of the administered dose present in liver at a given timepoint. The points indicated with an open circle "o" indicate the percent of the administered dose present in blood at a given timepoint. The points indicated with an open triangle "Δ" indicate the percent of the administered dose present in spleen at a given timepoint. The pharmacokinetics of PEG in blood are shown in Figure 1E. The areas under the curve $AUC|_0^{200}$ (mg•hour•ml^{-1} in the case of for blood, mg•hour•g^{-1} in the case of tissues) were calculated for blood, liver and spleen using linear trapezoidal rule *(Pharmacokinetics,* Ed. by M. Gibaldi, D. Perrier, Marcel Dekker, Inc. N.Y., Basel, 1982):

$$AUC_{tissue}|_0^{t_n} = C_1 \cdot t_1 + \Sigma[(C_{i-1} + C_i) \cdot \Delta t_i/2]$$

where C_i is the concentration in tissue measured in the *i*-th time interval, t_1 and t_n are the first and last collection times respectively, Δt_i is the time interval t_i-t_{i-1}. The partitioning coefficients ($P_{org/blood}$) were calculated as tissue to blood AUC ratio. The results are presented below:

Organ	L61		P85		F68		F108	
	$AUC\|_0^{200}$	$P_{org/blood}$	$AUC\|_0^{200}$	$P_{org/blood}$	$AUC\|_0^{200}$	$P_{org/blood}$	$AUC\|_0^{200}$	$P_{org/bloc}$
Blood	0.044	-	2.180	-	0.111	-	0.026	-
Liver	0.215	4.93	7.365	3.38	0.225	2.03	0.079	3.03
Spleen	0.118	2.68	4.240	1.94	0.066	0.59	0.032	1.23
Lungs			1.787	0.82				
Kidneys			1.748	0.80				
Heart			1.277	0.58				
Brain			0.377	0.17				

Example 9C - Block Copolymer Interactions with Blood Cells

1 μl of 1 % solutions of [^{3}H]-labeled Pluronic L61, P85 and F108, obtained as described above, were added to 100 μl of whole blood collected from female Balb/c mice. The samples were incubated for 30 min at 37°C, then transferred to 4°C and supplemented with 1 ml of cold PBS. The blood cells were then separated into erythrocytes, lymphocytes and plasma using density gradient centrifugation in 1 ml Histopaque. The amount of copolymer in the separated cell fractions was determined as described in Example 1. The results are presented in Fig. 2. The solid bars indicate the percentage of the radioactivity in the erythrocyte fraction. The striped bars indicate the percentage of the radioactivity in the plasma fraction. The open bars indicate the percentage of the radioactivity in the lymphocyte fraction.

Example 9D - Polymer biodistribution

Radioactive, tritium-containing derivatives of Pluronic P85 polymers were obtained from Kurchatov Institute of Atomic Energy, Moscow, Russia. 100 μl per 20 g of body weight of a 1% w/v isotonic solution of the radioactive copolymer (2 x 10^7 cpm/20g body weight) was administered *i.v.* into (a) BALB/c mice (from

Kriukovo Veterinary Dept. of Russian Acad. Medical Sciences, Moscow, Russia) and (b) BALB/c mice into which 3 x 10^6 SP2/0^{dnr} murine myeloma cells (described in Example 9A) had been injected subcutaneously 6 weeks previously. The biodistribution of polymer at various times post-injection of the radioactive copolymer was measured by sacrificing treated mice at the various timepoints, excising the tissues listed in the tables below, and quantifying the distribution of radioactivity by liquid scintillation counting. To prepare tissue samples for liquid scintillation counting, samples were placed in 1 ml of tissue solubilizer (available from Serva Chemicals, Germany) and homogenized in the cold. The homogenates were incubated for 14 hours at room temperature, decolorized with 50 μl of 30% hydrogen peroxide, and incubated overnight at room temperature.

For BALB/c mice lacking injected tumor cells, the results were:

Organ	Polymer content (% of initial dose per organ)		
	73 hours	92.5 hours	121 hours
Spleen	0.23	0.2	0.12
Liver	3.69	3.27	1.8

For BALB/c mice with injected tumor cells, the results were:

Organ	Polymer content (% of initial dose per organ)		
	73 hours	92.5 hours	121 hours
Spleen	0.35	0.47	0.36
Liver	3.71	3.35	3.35
Tumor	1.53	6.24	1.50

Additional observations derived from this set of experiments were (1) that degradation products of the polymers were not observed until 24 hours after polymer administration and (2) complete clearance of polymer from the mice occurred 250 to 300 hours after administration.

Example 9E - Blood concentrations of copolymer

100 μl/20g body weight of the [^{3}H]-Pluronic P85 of Example 9A (at 0.2% concentration) were administered to 3 to 4-week old BALB/c mice by *i.v.* injection or orally. The amount of radioactivity (cpm x 10^{-3}/10μl) found in the blood of the mice at various timepoints post injection is shown in Figure 3A, where line 1 is for *i.v.* injected polymer, and line 2 is for orally administered polymer. Corresponding data (cpm/10mg) are shown for spleen (Fig. 3B), brain (Fig. 3C), liver (Fig. 3D) and kidney (Fig. 3E).

Example 9F - Acute Toxicity of Copolymers

The acute toxicity of Pluronic F108, P85 and L61 were studied in 5-week old BALB/c male mice. Each experimental group of mice included 6 mice. Various doses of isotonic Pluronic solutions were administered *i.p.* Animal mortality was monitored daily for 14 days. The LD_{50} and maximum tolerated dosage ("MTD", i.e., the maximal dose at which no animals among 6 equivalently treated animals died) were calculated by probit analysis. *See*, Chan and Hayes in *Principles and Methods of Toxicology*, Hayes, A.W., ed., Raven Press, New York, 1989, pp. 169-189. The results were as follows:

Pluronic	MTD, g/kg	LD_{50}, g/kg
Pluronic L61	0.1	0.8
Pluronic P85	0.2	0.8
Pluronic F108	5.0	9.0

Example 10 - Serum Levels Over Time

Either Conjugate A** (containing a tracer amount of [^{3}H]daunorubicin) or free [^{3}H]daunorubicin, was injected intraperitoneally into a number of 7 week old C57B1/6 male mice at a dose of 5 mg/kg body weight (based on daunorubicin content). At a given time, the animals in each treatment group were sacrificed and the amount of radioactivity in the blood determined. Twenty-four mice were used for each treatment group. The results were as follows:

Time after drug administration	Drug in Plasma (cpm μl of blood)	
	Conjugate A	Daunorubicin
5 min	23(±3)	431(±53)
10 min	42(±5)	442(±58)
30 min	145(±17)	360(±49)
1 h	276(±19)	130(±22)
3 h	423(±39)	60(±9)
9 h	568(±65)	17(±5)
15 h	710(±68)	0
20 h	725(±76)	0
30 h	606(±53)	0
40 h	281(±31)	0
55 h	129(±16)	0
75 h	98(±11)	0

Example 11 - Tumor Treatment

Six week old female Balb C mice were inoculated subcutaneously with 3×10^6 Sp2-resistant myeloma cells. Note that these cells, when injected into mice to create solid tumors, retain multi-drug resistance even 50 days after inoculation into the mice. Conjugate A or free daunorubicin was injected intravenously at 14, 18 and 22 days after tumor inoculation. The amount of conjugate A per and of free daunorubicin administered per injection was the MTD determined in Example 8. Tumor volumes were calculated by multiplying the long diameter of the tumor by the short diameter. The observed results were normalized by dividing the volume at a given day post-treatment (V) by the volume observed on the first day of treatment (V_0). The results were as follows:

Days after first treatment	V/V_0		
	Conjugate A	Treated Control	Untreated Control
0	1	1	1
●	1.5	2.7	2.5
6	2.1	3.4	3.2
8	2.9	5.1	5.5
10	4.2	6.6	8.4
13	5.6	11.6	12.1
16	8.6	25.9	18.6
20	10.1	25.9	25.6
23	13.8	38.4	32.8
27	17.1	50.9	42.4
31	16.9	68.3	45.5
34	18.2	73.0	61.4
38	19.4	83.4	73.4
42	21.1	113.8	100.6
45	20.5	111.9	123.6

Example 12 - Tumor Treatment

The same procedures outlined for Example 11 were utilized, except that the Sp2 myeloma cell line, which is not multi-drug resistant, was used. The results were as follows:

Days after first treatment	V/V_0		
	Conjugate A	Treated Control	Untreated Control
0	1	1	1
4	2.6	1.5	2.3
7	3.2	2.4	4.1
12	5.0	8.8	6.3
14	5.1	10.9	9.4
17	6.3	16.8	12.5
21	8.2	17.5	16.5
25	8.9	16.1	18.5
28	9.0	15.8	18.4
32	9.4	15.9	20.1
35	5.4	15.7	21.1
40	5.2	--	--
45	4.5	--	--

Example 13 - Compositions for I.P. Administration

Conjugate A (900 mg) and conjugate B were separately dissolved in 100 mL RPMI 1640. The solutions were heated for 30 minutes at 37°C, and then filtered through a 0.22 μm filter.

These compositions can be stored in the dark at room temperature for 7 days without substantial loss of cytotoxicity. Alternately, the compositions can be lyophilized and stored for at least 1 year in the dark at room temperature.

Example 14 - Composition Suitable for I.P. Administration

An ascorbate solution was prepared by dissolving 100 mg of sodium ascorbate and 100 mL of 9% sodium chloride. Conjugate B (500 mg) was dissolved in the ascorbate solution. The solution was incubated for 30 minutes at 37°C, and then filtered through a 0.22 μm filter. The composition was suitable for storage in the dark at room temperature for 7 days. Alternately, it can by lyophilized and stored for at least 1 year in the dark at room temperature.

Example 15 - Micellar Agent Delivery

Micelles were formed with a 1:1.5 (w/w) mixture of two polyoxyethylene-polyoxypropylene block copolymers of formula (I), wherein ratios (n) for the polymers were 1.00 and 1.50, respectively. These copolymers were formulated at 2.0% (w/v) in RPMI 1640 medium, and the mixture filter sterilized. To create a stock solution of a biological agent dissolved in the micelles of this formulation, daunorubicin was added to the copolymer solution.

The cytotoxicity of free daunorubicin and micelle dissolved daunorubicin was tested against (1) the MCF-7 line of human breast cancer cells, (2) the MCF-7AU line derived therefrom, which cell line is daunorubicin resistant but does not express the P-170 marker protein associated with some forms of drug resistance, and (3) the Dox-MCR-7, a MCF-7 derived cell line that is drug resistant and expresses the P-170 marker protein. The results were as follows:

Daunorubicin conc. (ng/mL):		50,000	10,000	2,000	400	80	16
	Cell line	% Inhibition					
Micelle formulation	MCF-7	100	100	84	65	42	12
	MCF-7AU	100	100	100	96	89	39
	Dox-MCF-7	100	100	100	89	73	45
Free drug	MCF-7	100	100	91	69	43	15
	MCF-7AU	100	89	65	37	9	3
	Dox-MCF-7	100	86	62	39	7	2

Example 16 - Conjugate Synthesis with Oligonucleotide

A 12-mer oligonucleotide, 5′-CGTTCCTCCTGU ("Oligo A") complimentary to the splicing site (positions 983-994 on the viral genome) of the early mRNA of type 1 Herpes SimplexVirus ("HSV-1"), was synthesized using a 380B-02 DNA-synthesizer (Applied Biosystems, CA). The synthesizer used phosporamidite chemistry and an 8 min. synthesis cycle. Cycle conditions and preparation of the crude product were done as recommended by Applied Biosystems. The crude Oligo A obtained from the synthesis was precipitated from a 1 M LiCl solution (0.5 ml) with acetone (2 ml). The precipitate was dissolved in triethylammonium acetate buffer and purified by reverse-phase high performance liquid

chromatography on a Silasorb C18 column (9X250 mm, Gilson, France) developed with an acetonitrile gradient in a 20 mM TEAA buffer (pH 8.5).

The 3′-terminal of the purified Oligo A was oxidized with periodate to create an aldehyde and conjugated by reductive alkylation with a hexamethylene-diamine linker, creating an amine derivative. See Che-Chung et al., *Biochem. Internat.*, 25:767 (1991); Vinogradov et al., *BBRC*, 203:959 (1994). Pluronic A was similarly oxidized to create terminal aldehydes. The amine derivative (1 mg) was dissolved in 100 μl of 0.1 M borate buffer (pH 9.0) and mixed with 2 mg of the Pluronic A derivative. 1.5 mg of sodium cyanoborohydride was added to the mixture to reduce the Schiff's bases formed between the amine and aldehyde groups. This reaction was allowed to proceed for 12 hours at 4°C. The polymeric product of this reaction was isolated by gel filtration chromatography on Sephadex LH-20, utilizing 90% aqueous isopropanol as the eluent. The conjugate so obtained is referred to hereinafter as "Oligo A Conjugate."

Example 17- The Effect of Oligo A Conjugate on Virus Production

Oligo A and Oligo A Conjugate were separately dissolved in RPMI 1640 medium (ICN Biomedicals Inc., Costa Mesa, CA) to a final concentration of 0.2 mM (based on oligonucleotide absorbance). These stock solutions were then filtered through 0.22 μm filters to remove any possible bacterial or fungal contamination.

Monolayers of Vero cells were incubated for 1 hour at 37°C in serum-free RPMI 1640 together with various concentrations of Oligo A or Oligo A Conjugate. The monolayers, while still exposed to oligonucleotides, were then infected with 1 plaque forming unit per cultured cell of HSV-1, strain L2 (from the Museum of Virus Strains of the D.I. Ivanovskii Institute of Virology, Russian Academy of Sciences, Russian Federation). This infection method has been described by Vinogradov et al., *BBRC*, 203:959 (1994). After 8 hours of exposure to virus and oligonucleotides, the medium on the cells was replaced with fresh medium containing 10% FCS. Medium from the cells was collected at 22 and 39 hours after the infective incubation, and the virus titer in the collected medium was

determined as described in *Virology, A Practical Approach*, Mahy, Ed., IRL Press, Oxford Univ. Press, Washington, DC, 1985. The results were as follows:

Sample concentration (mM)	Oligonucleotide concentration (μM)	Infectious Titer of HSV-1 (PFU/ml)	
		22 hours past infection	39 hours past infection
Control (cells without oligonucleotides	0	$5x10^6$	$1x10^7$
Oligo A	10	$3x10^6$	$5x10^6$
	5	$5x10^6$	$1x10^7$
	2	$5x10^6$	$1x10^7$
	1	$5x10^6$	$1x10^7$
Oligo A Conjugate	10	0	0
	5	0	$5x10^2$
	2	$1x10^3$	$7x10^3$
	1	$5x10^4$	$3x10^6$

Example 18 The Activity of Conjugated TNFα

Pluronic A (1 mg) that had been oxidized to create terminal aldehyde groups and 0.3 mg of human tumor necrosis factor α ("TNFα") (from ICN Biomedicals Inc., Costa Mesa, CA) were incubated together in 0.4 ml of 0.1 M borate buffer (pH 9.0). The Schiff's bases that formed between the peptide hormone's amino groups and the aldehydes of Pluronic A were reduced by adding 0.6 mg of sodium cyanoborohydride and incubating for 12 hours at 37°C. See Kabanov et al., *J. Contr. Release*, 22: 141 (1992). The TNFα conjugate so obtained was isolated by gel filtration on Sephadex G-25 utilizing phosphate buffered saline, pH 7.0, as the eluent. The peptide-based concentration of the isolated conjugate and of comparative solutions of unconjugated TNFα were determined spectrophotometrically at 280 nm.

The specific activity of the conjugated and unconjugated TNFα with respect to SK cells were determined by XTT analysis of mitochondrial activity (see Example 5, above). The cells were exposed to various concentrations of conjugated or unconjugated TNFα for 24 hours. The cells were then washed three times with RPMI 1640 medium and analyzed by XTT analysis. The results were as follows:

TNFα concentration, (nM)	Inhibition, % (±SD)	
	TNFα - Conjugate	TNFα
0.01	9(±4)	0
0.05	15(±5)	2(±2)
0.2	35(±6)	5(±3)
1.0	74(±9)	4(±4)
5	91(±11)	10(±5)
20	100	22(±5)
50	100	35(±7)
100	100	44(±6)
200	100	80(±14)

What is claimed is:

1. A conjugate between a biologically active agent and a block copolymer comprising a biologically active agent covalently linked to at least one end group of a block copolymer comprising (1) an A-type linear polymeric segment comprising repeating units which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500 and (2) a B-type linear polymeric segment comprising repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units for each said polymeric segment comprise an ether linkage.

2. The conjugated biologically active agent of claim 1 wherein said block copolymer comprise a polymer of formulas

A-B-A′, A-B, B-A-B′, or **L(R^1)(R^2)(R^3)(R^4)**

(I) (II) (III) (IV)

wherein A and A′ are A-type linear polymeric segments, wherein B and B′ are B-type linear polymeric segments, and wherein R^1, R^2, R^3 and R^4 are (1) block copolymers of formulas (I), (II) or (III) or (2) hydrogen and L is a linking group, with the proviso that no more than two of R^1, R^2, R^3 or R^4 shall be hydrogen.

3. The conjugated biologically active agent of claim 1 wherein 90% of the linkages joining the repeating units for each said polymeric segment comprise ether linkages.

4. The conjugated biologically active agent of claim 1 wherein said agent comprises a anthracycline, vinca alkaloid, mitomycin-type antibiotic, bleomycin-type antibiotic, fluconazole, amphotericin B, paclitaxel, taxotere and related compounds and derivatives, immunomodulator or cytokine, erythropoietin, or polynucleotide.

5. The conjugated biologically active agent of claim 1, wherein the biologically active agent is selected from the group consisting of doxorubicin, daunorubicin, carminomycin, fluconazole, amphotericin B, and mixtures thereof.

6. The conjugated biologically active agent of claim 1 wherein the repeating units for each said polymeric segment have molecular weight between about 30 and about 100.

7. The conjugated biologically active agent of claim 6 wherein 90% of the linkages joining the repeating units for each said polymeric segment comprise ether linkages.

8. The conjugated biologically active agent of claim 7 wherein 95% of the linkages joining the repeating units for each said polymeric segment comprise ether linkages.

9. The conjugated biologically active agent of claim 8 wherein all of the repeating units that comprise blocks B or B′ have a Hansch-Leo fragmental constants of about -.30 or more.

10. The conjugated biologically active agent of claim 9 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.4 or less.

11. The conjugated biologically active agent of claim 10 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.5 or less and the repeating units that comprise blocks B or B′ have Hansch-Leo constants of about -0.2 or more.

12. The conjugated biologically active agent of claim 11 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.7 or less.

13. The conjugated biologically active agent of claim 1 wherein the repeating units for each said polymeric segment consists essentially of repeating units of formula -O-R-, wherein R is a C1 to C6 alkyl group.

14. The conjugated biologically active agent of claim 13 wherein all of the repeating units that comprise blocks B or B′ have a Hansch-Leo fragmental constants of about -.30 or more.

15. The conjugated biologically active agent of claim 14 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.4 or less.

16. The conjugated biologically active agent of claim 15 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.5 or less and the repeating units that comprise blocks B or B′ have Hansch-Leo constants of about -0.2 or more.

17. The conjugated biologically active agent of claim 16 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.7 or less.

18. The conjugated biologically active agent of claim 1 wherein the repeating units for each said polymeric segment consists essentially of repeating units of formula -O-R^5, wherein n is an integer from 0 to about 5 and R^6 is R^5 is

(1) -$(CH_2)_n$-$CH(R^6)$-, wherein n is an integer from 0 to about 5 and R^6 is hydrogen, cycloalkyl having 3-8 carbon atoms, alkyl having 1-6 carbon atoms, phenyl, alkylphenyl wherein the alkyl has 1-6 carbon atoms, hydroxy, hydroxyalkyl wherein the alkyl has 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, an alkylcarbonyl group having 2-7 carbon atoms, alkoxycarbonyl wherein the alkoxy has 1-6 carbon atoms, alkoxycarbonylalkyl wherein the alkoxy and alkyl each independently has 1-6 carbon atoms, alkylcarboxyalkyl wherein each alkyl group has 1-6 carbon atoms, aminoalkyl wherein the alkyl group has 1-6 carbon atoms, alkylamine or dialkylamino wherein each alkyl independently has 1-6 carbon atoms, mono- or di-alkylaminoalkyl wherein each alkyl independently has 1-6 carbon atoms, chloro, chloroalkyl wherein the alkyl has from 1-6 carbon atoms, fluoro, fluoroalkyl wherein the alkyl has from 1-6 carbon atoms, cyano, cyano alkyl wherein the alkyl has from 1-6 carbon atoms or carboxyl;

(2) a carbocyclic group having 3-8 ring carbon atoms, which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents, or

(3) a heterocyclic group, having 3-8 ring atoms, which can include heterocycloalkyl or heteroaromatic groups, and which can include from 1 to 4 heteroatoms selected from the group consisting of oxygen, nitrogen, sulfur

and mixtures thereto, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents.

19. The conjugated biologically active agent of claim 20 wherein all of the repeating units that comprise blocks B or B′ have a Hansch-Leo fragmental constants of about -.30 or more.

20. The conjugated biologically active agent of claim 21 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.4 or less.

21. The conjugated biologically active agent of claim 22 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.5 or less and the repeating units that comprise blocks B or B′ have Hansch-Leo constants of about -0.2 or more.

22. The conjugated biologically active agent of claim 21 wherein all of the repeating units that comprise blocks A or A′ have a Hansch-Leo fragmental constants of about -0.7 or less.

23. The conjugated biologically active agent of claim 18 wherein at least about 80% by weight of the repeating units that comprise blocks B or B′ are propylene oxide repeating units and at least about 80% by weight of the repeating units that comprise blocks A or A′ are ethylene oxide repeating units.

24. A method of delivering a conjugated biologically active agent orally comprising orally administering the conjugate between a biologically active agent and a block copolymer of claim 1.

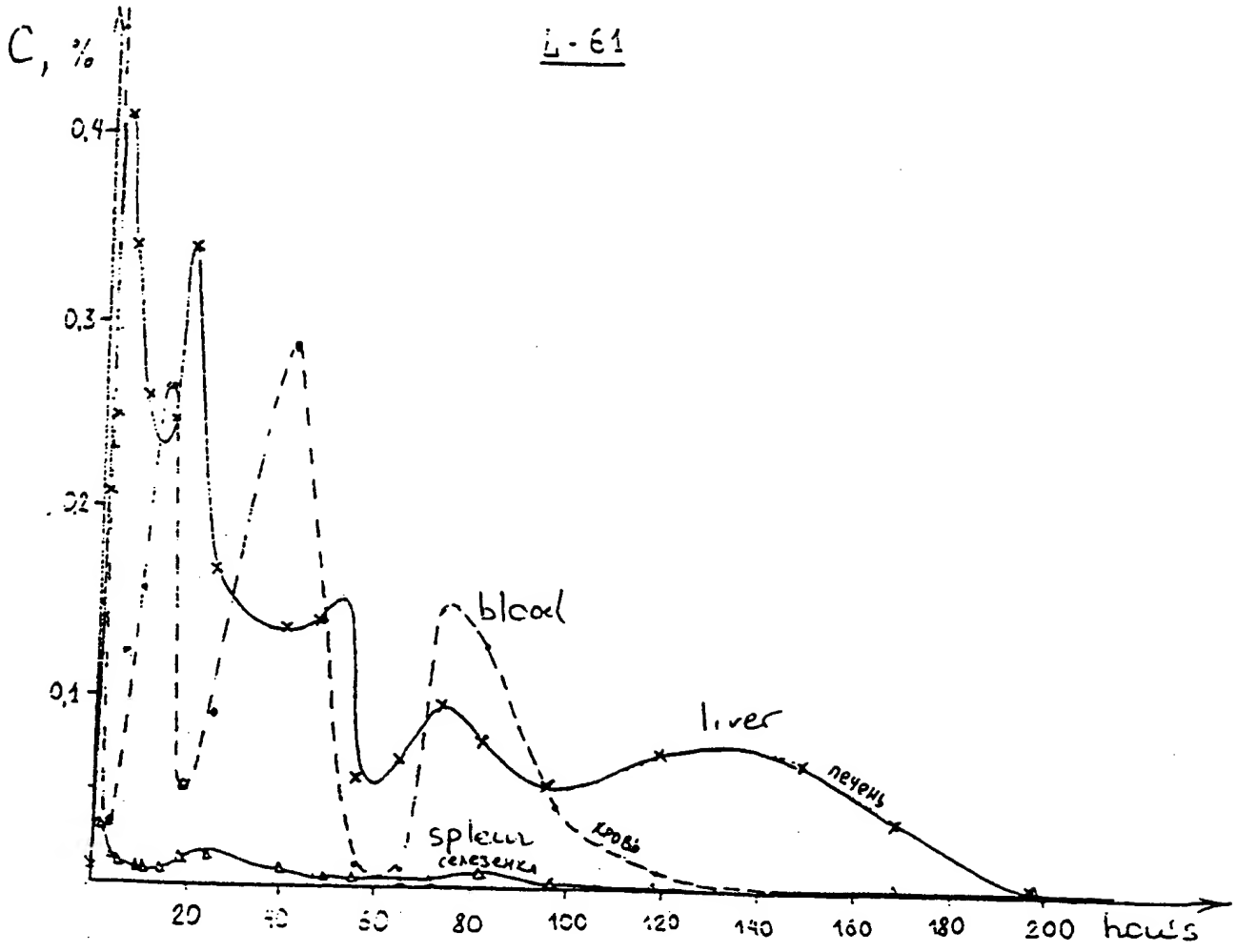

C, %
L-61
0,4
0,3
0,2
0,1
blood
liver
печень
spleen
селезенка
20
40
60
80
100
120
140
160
180
200
hours

Fig 1a

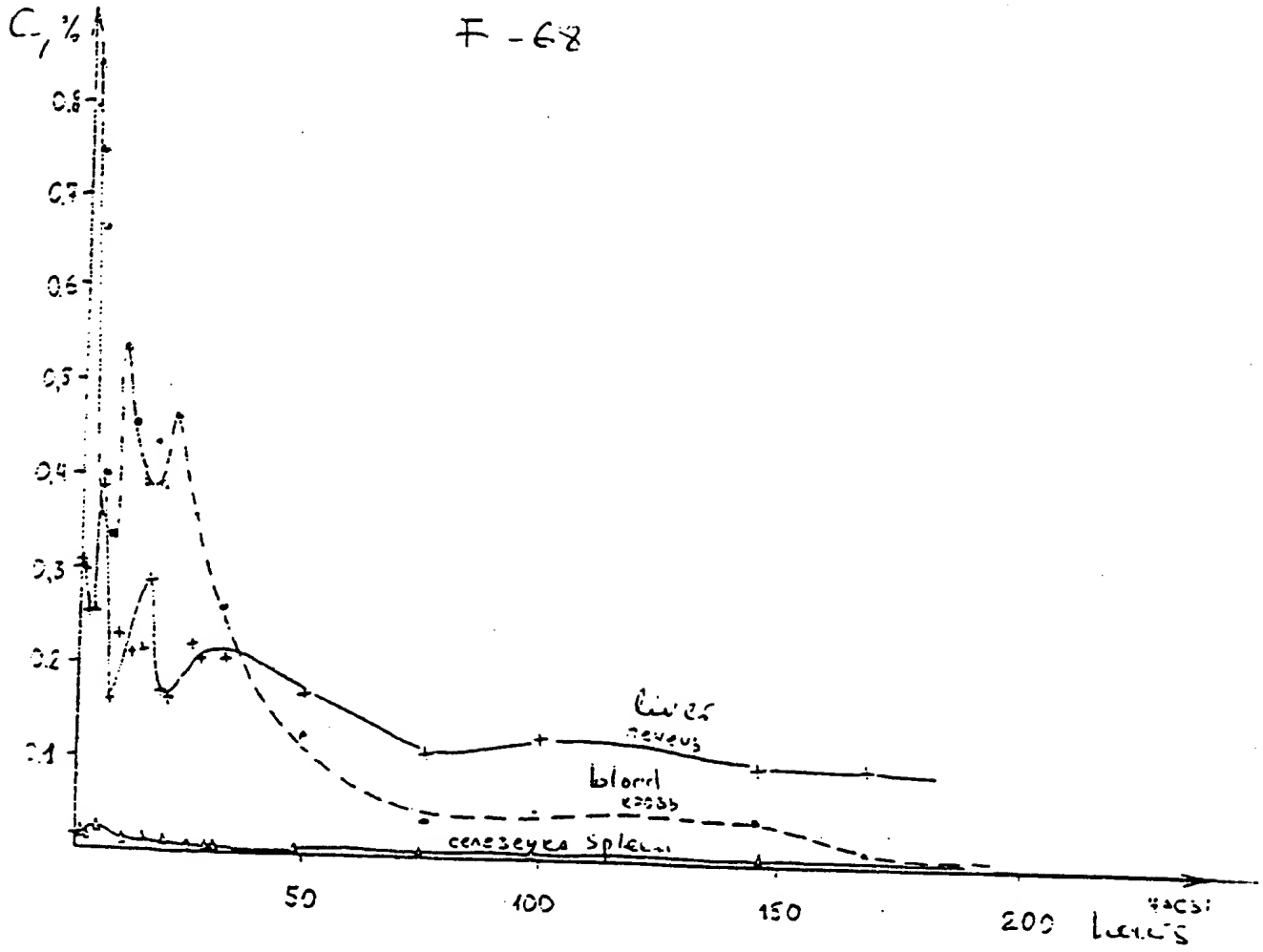


Fig 1b

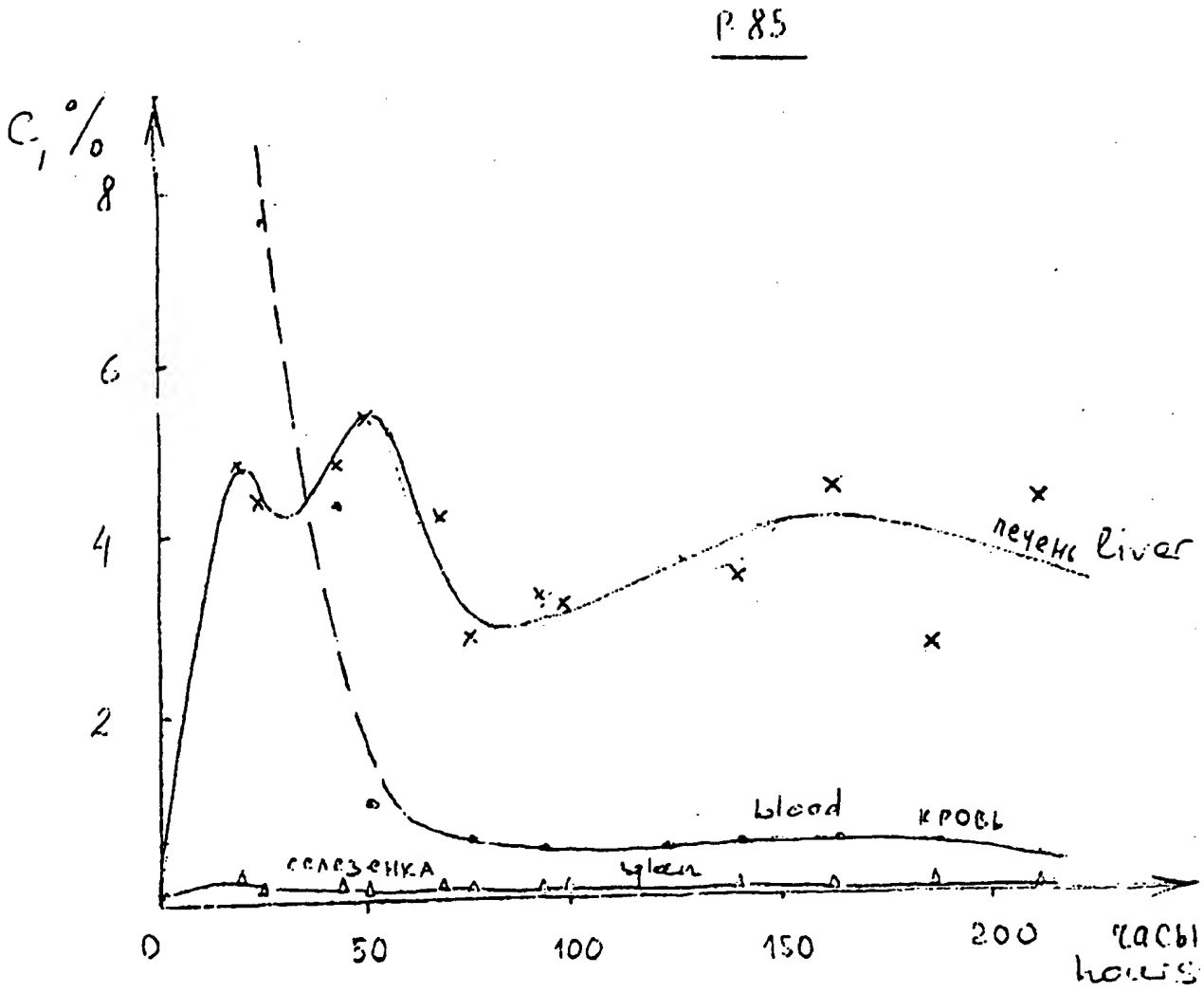

P 85
C, %
8
6
4
2
печень liver
Blood
кровь
селезенка
spleen
0
50
100
150
200
часы
hours

Fig 1c

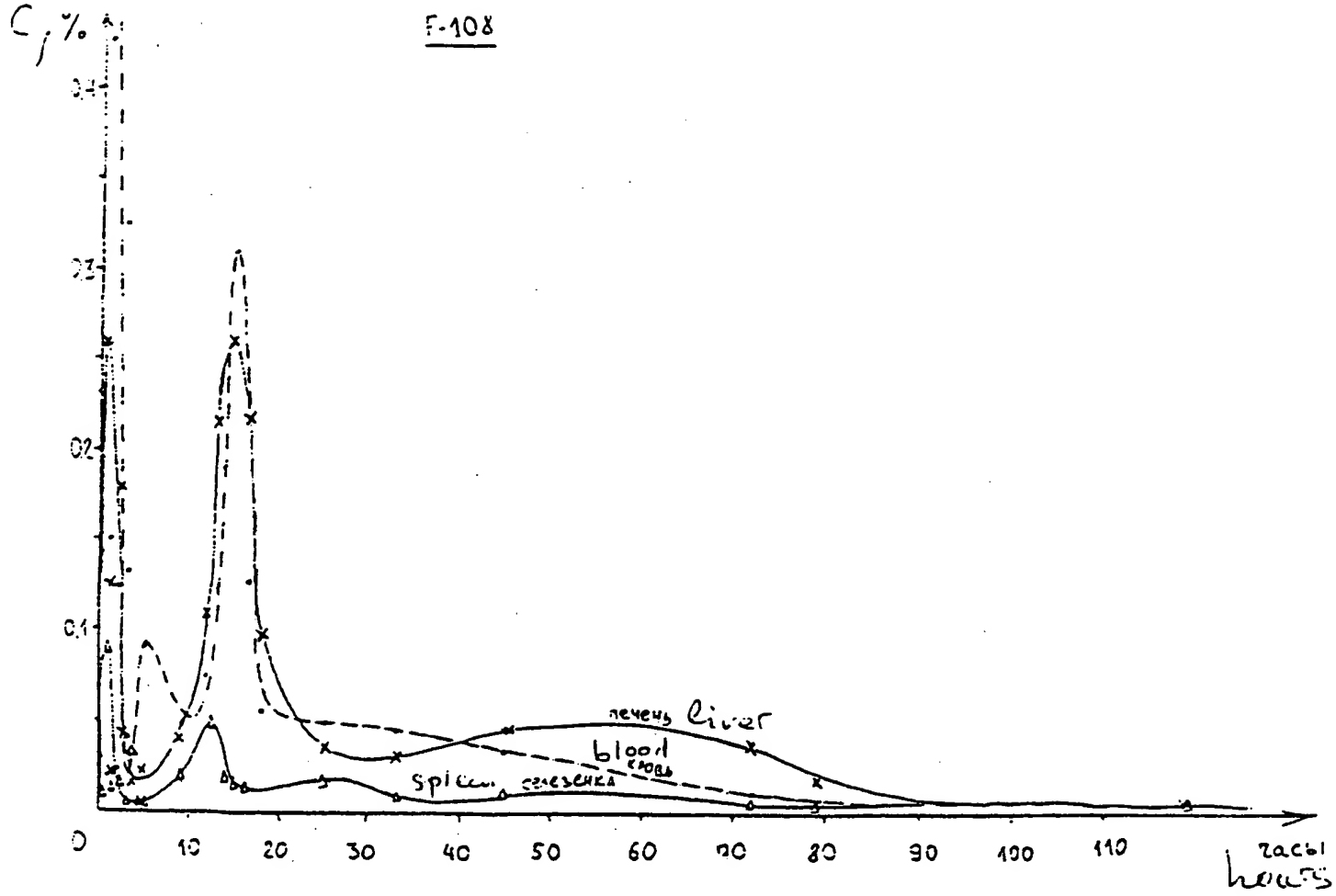

C, %
F-108
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
90
100
110
часы
hours
печень liver
blood кровь
spleen селезенка

Fig 1d

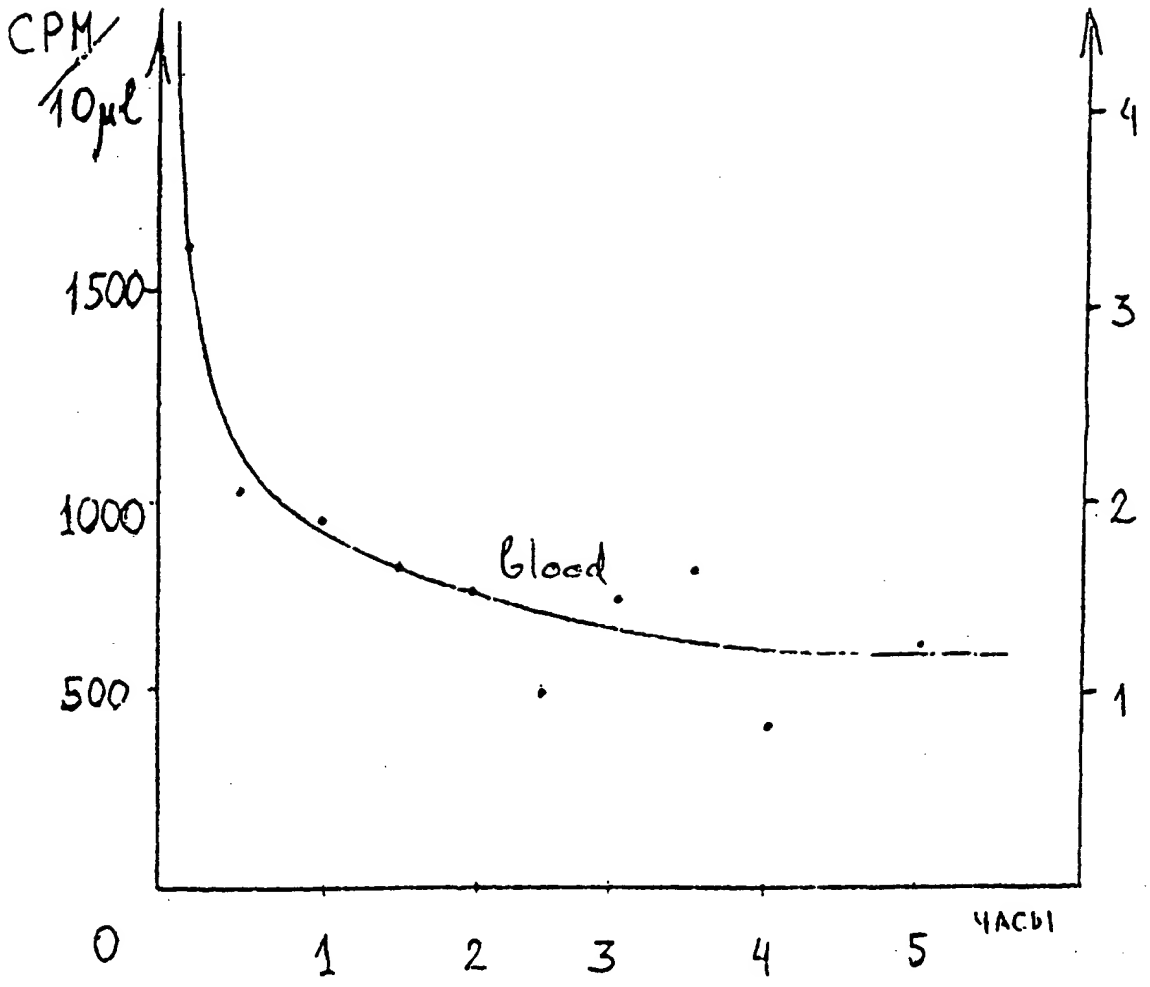


Fig 1e-

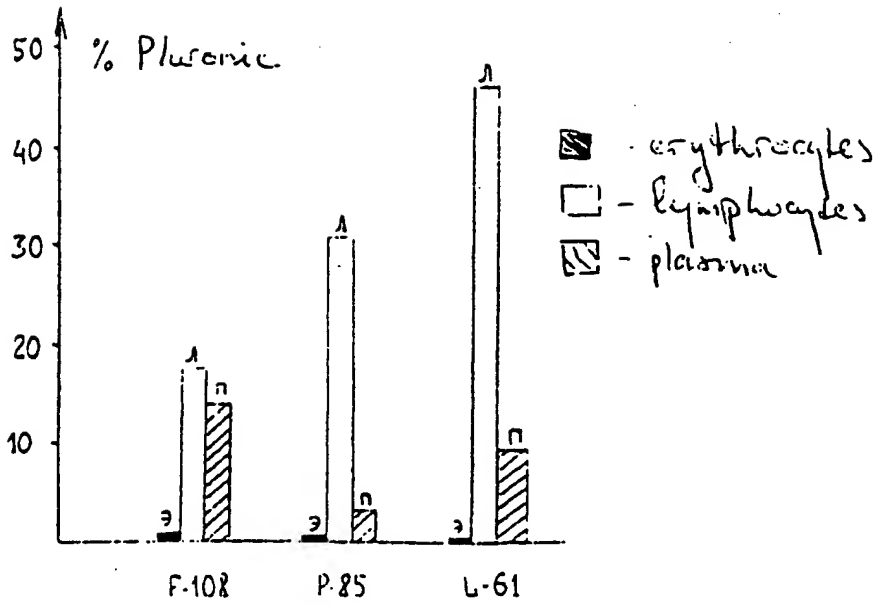

50
40
30
20
10
% Pluronic
- erythrocytes
- lymphocytes
- plasma
F-108
P-85
L-61

Fig. 2

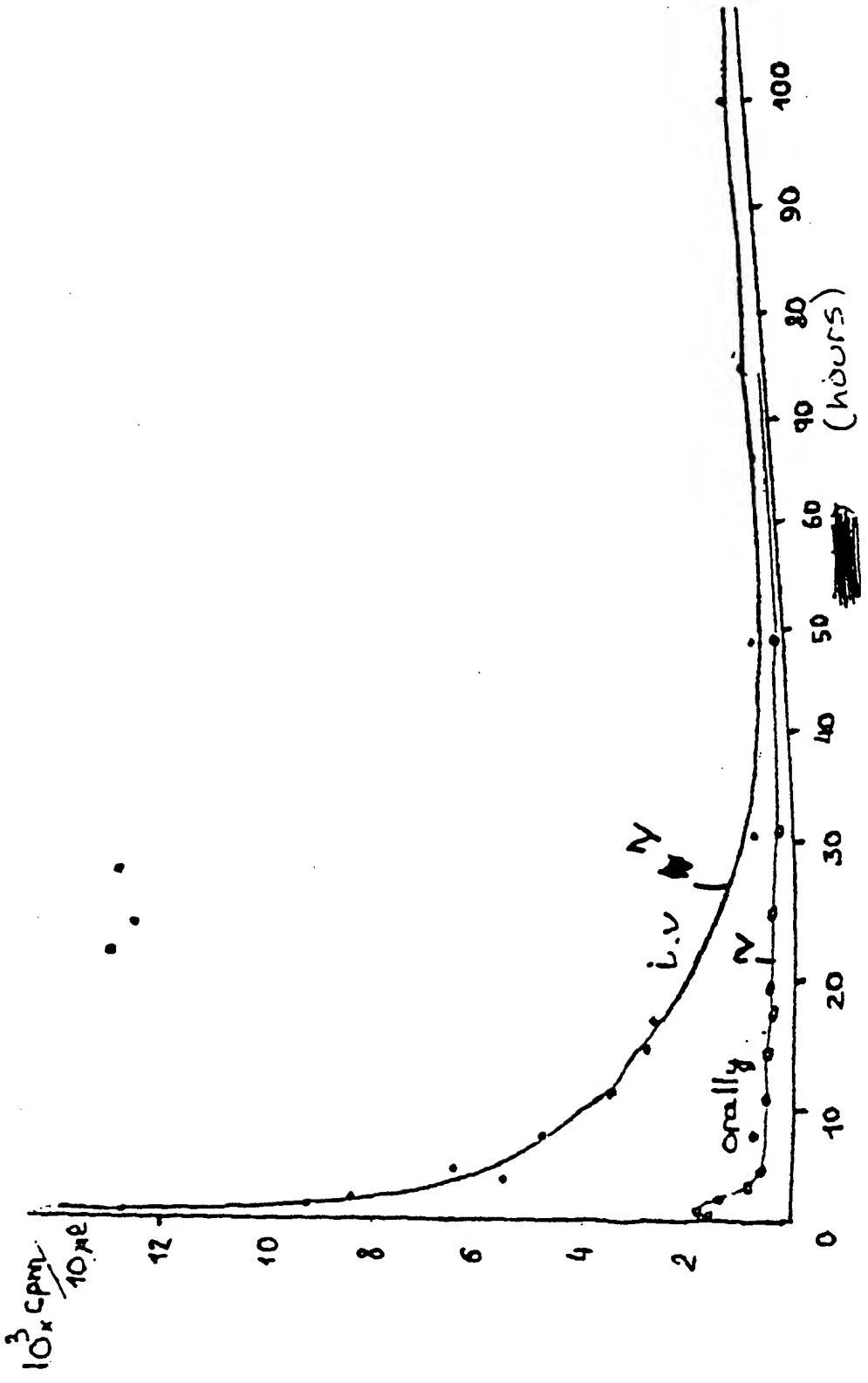

10^3 cpm/10μl
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
100
(hours)
i.v
orally

Fig 3a

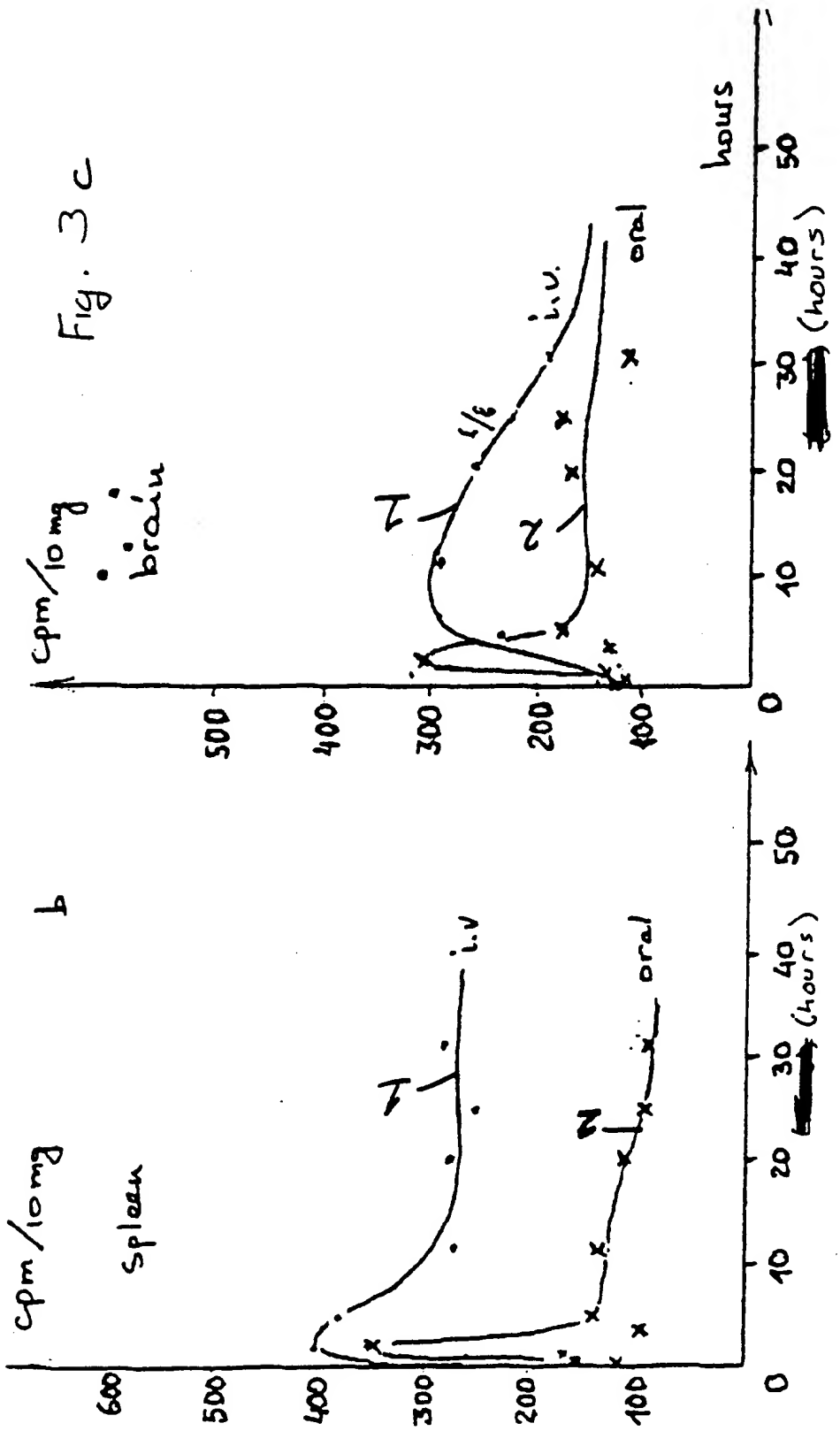
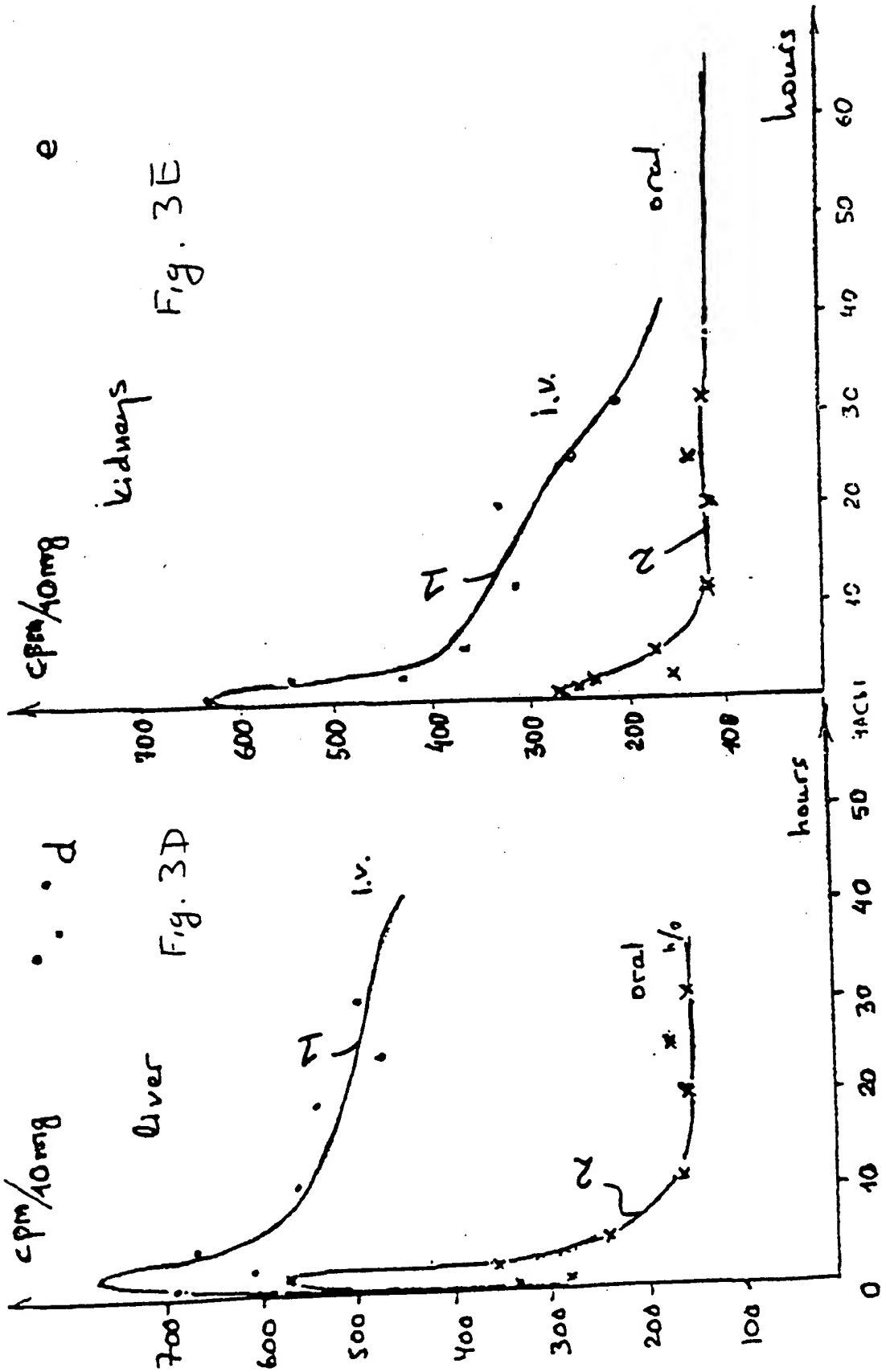


Fig. 3c
cpm/10mg
brain
i.v.
oral
hours
(hours)
Fig 3b
b
cpm/10mg
spleen
i.v
oral
(hours)


e
Fig. 3E
kidneys
cpm/10mg
i.v.
oral
hours
d
Fig. 3D
liver
cpm/10mg
i.v.
oral
hours

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, medline, biosis, ca, embase
Searc Terms: kabanov?/au; alakhov?/au; conjugat?; block; copolym?; hansch; leo; link?; antibiotic; gene; therap?; deliv?; heterocyc?

INTERNATIONAL SEARCH REPORT

Inter[illegible]ional application N[illegible]
[illegible]/US95/15084

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 27/00, 43/00, 61/00; A61K 31/00, 31/01, 31/70

US CL :514/2, 44, 762

According to International Patent Classificati n (IPC) r to both national classificati n and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classificati n system foll wed by classificati n symbols)

U.S. : 514/2, 44, 762

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCONJUGATE CHEMISTRY, Volume 4, issued 1993, Kabanov et al., "Efficient Transformation of Mammalian Cells Using DNA Interpoiyelectrolyte Complexes with Carbon Chain Polycations", pages 448-454, see entire document.	1-24
Y	JOURNAL OF CONTROLLED RELEASE, Volume 22, issued 1992, Kabanov et al., "A new class of drug cariers; micelles of poly(oxypropylene) block copolymers as microcontainers for drug targeting from blood in brain", pages 141-158, see entire document.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 MAY 1996	Date of mailing of the international search report 15 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15084

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	BIOCONJUGATE CHEMISTRY, Volume 6, Number 6, issued November/December 1995, Kabanov et al., "Water-Soluble Block Polycations as Carriers for Oligonucleotide Delivery", pages 639-643, see entire document.	1-24